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ENDOCRINOLOGY

VOLUME 49 DECEMBER, 1951 NUMBER 6

THE ANTAGONISTIC RELATIONSHIP BETWEEN TESTOSTERONE AND THYROXINE IN MAINTAINING THE EPIDERMIS OF THE MALE RAT

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IN PREVIOUS work on the testis and thyroid hormones, attention has been focussed mainly on the androgenic function of testosterone and the metabolic function of thyroxine. Recently, it was found that physiological amounts of testosterone and thyroxine were essential for the maintenance of the normal weight and histological picture of such organs as heart, kidney, hypophysis and submaxillary glands in the male rat (Grad and Leblond, 1949). Thus, in the absence of testis and thyroid gland, there was an atrophy of the serous tubules of the submaxillary gland, while daily injection of replacement amounts of testosterone and thyroxine restored normal size and granular content of these tubules. In this case, the normalcy was achieved through a *synergistic* action of the two hormones. In the case of the hypophysis, a normal picture was obtained by an *additive* action of the two hormones. That at the same time these hormones may act *antagonistically* on other end organs was demonstrated in the present study of the epidermis. Testosterone was found to increase, and thyroxine to decrease the thickness of the epidermis in castrated-thyroidectomized rats, while their joint action resulted in an intermediate thickness approximating that in intact animals. Presumably, the thickness of the epidermis in the normal male rat is due, at least partly, to an interaction of the testis and thyroid hormones.

METHODS AND TECHNIQUES

Three similar experiments were conducted, using castrated-thyroidectomized male rats of 125–200 gm., fed Remington's low iodine diet No. 342, enriched with 10% yeast. There were at least four groups of animals in each experiment; 1) untreated control; 2) testosterone-treated (two subcutaneous injections of 250 μg. daily); 3) thyroxine-treated (two subcutaneous injections of 2.5 to 4 μg. daily, the dose being adjusted until

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a normal metabolic rate was obtained); 4) testosterone and thyroxine treated, with the above mentioned doses. In addition, there was a fifth group of normal intact animals (sham-operated and untreated) in the second and third experiments.

The first experiment consisted of the castrated-thyroidectomized animals described in a previous paper (Grad and Leblond, 1949). Treatment was continued for 47 days and at autopsy pieces of skin were taken from the animals' backs. In the other experiments, treatment was continued for 42 days and the pieces of skin were taken from the sides of the abdomen. Either area was sufficiently far from the injection site—at the base of the neck—to avoid possible interferences from the injection. Each piece of skin, measuring approximately 1 ×2 cm. was stuck onto a piece of cardboard and fixed in Orth for histological study. The sections were cut at 5μ and stained with hematoxylin-eosin.

The two main portions of the epidermis, namely, the deeper or Malpighian layer (including stratum germinativum and granulosum) and the outer or cornified layer were examined microscopically, and their thickness was assessed subjectively and expressed as a number of "+" (Table 1). Since the subjective results were similar in all three experiments, an objective histometric study was carried out in the first one only.

Direct measurements of the width of the epidermis were not done, due to large variations even within the same microscopic fields. A more precise technique was employed: sections of epidermis magnified 430 times were projected onto a sheet of white loose-leaf paper by a 45° prism adapted to the eyepiece of the microscope. The outlines of the epidermis in 6 consecutive fields were then traced on the paper; that is, the basement membrane and the outer limit of the cornified layer were drawn. The limit between

TABLE 1. EFFECT OF TESTOSTERONE AND THYROXINE ON THE THICKNESS OF THE EPIDERMIS IN CASTRATED-THYROIDECTOMIZED RATS (SUBJECTIVE RESULTS)

	Control	Testosterone	Thyroxine	Testosterone and thyroxine	Normal control (sham operated)
Experiment 1					
No. of animals in group	4	5	7	8	
Mean body weight (gm.)					
Initial	175	180	168	175	
Final	168	163	213	206	
Mean thickness (in ++)					
Malpighian layer	2.2	3.4	1.0	2.1	
Cornified layer	2.5	4.2	1.3	3.1	
Experiment 2					
No. of animals in group	8	8	5	3	15
Mean body weight (gm.)					
Initial	134	134	132	133	133
Final	156	168	226	206	258
Mean thickness (in ++)					
Malpighian layer	1.7	2.2	1.8	1.0	1.9
Cornified layer	2.2	4.1	1.4	2.0	2.9
Experiment 3					
No. of animals in group	7	7	5	5	6
Mean body weight (gm.)					
Initial	145	159	172	152	173
Final	162	165	226	218	227
Mean thickness (in ++)					
Malpighian layer	2.1	2.9	1.8	2.7	1.4
Cornified layer	2.7	4.0	0.8	2.5	1.5

TABLE 2. EFFECT OF TESTOSTERONE AND THYROXINE ON THE THICKNESS OF THE EPIDERMIS IN CASTRATED-THYROIDECTOMIZED RATS

	Control	Testosterone	Thyroxine	Testosterone and thyroxine
Mean Thickness (μ)				
Whole epidermis	50.7	103.9	31.3	79.6
Malpighian layer	23.2	28.9	16.4	25.2
Cornified layer	27.3	75.0	14.9	54.4

Statistical Analysis: P Values

	Control vs. testosterone	Control vs. thyroxine	Control vs. testosterone and thyroxine	Testosterone vs. thyroxine	Testosterone vs. testosterone and thyroxine	Thyroxine vs. testosterone and thyroxine
Whole epidermis	0.01	0.10	0.02	0.001	0.05	0.001
Malpighian layer	0.10	0.05	0.90	0.001	0.10	0.01
Cornified layer	0.01	0.20	0.02	0.001	0.05	0.001

Malpighian and cornified layer was also indicated. By cutting along the drawn lines, pieces of paper corresponding to the two main layers of the epidermis were obtained. They were then weighed on an analytical balance, thus yielding the relative weights of these two layers.

The surface area corresponding to 1 mg. of paper was then calculated by weighing pieces of paper of known area. This made it possible to reëxpress the relative weights of the epidermal layers as surface areas, which, after dividing by the square of the magnification, gave the actual surface area of each layer in the 6 fields drawn.

The total length of the basement membrane in the 6 fields was then measured by following the outline of its projection with a map reader and dividing the reading by the magnification. Assuming that the surface area of the projected Malpighian layer is a rectangle, the base of which is formed by the basement membrane, the mean thickness of this layer was readily obtained by dividing its area by the length of the basement membrane. The mean thickness of the cornified layer was similarly determined by assuming that its projection was a rectangle of length equal to the basement membrane. The mean thickness of the epidermis was finally obtained by adding the figures for Malpighian and cornified layers (Table 2).

Since the differences in epidermal thickness might be due to differences in the mitotic activity of the epidermal cells, metaphases and anaphases were counted under oil immersion along the whole length of the sections of epidermis. The total length of the basement membrane was then estimated by projecting the sections at a magnification of 38 times, tracing the basement membrane on paper, and determining its length with the map reader. The results were expressed as number of mitoses per centimeter of basement membrane (Table 3). This method of expressing the mitotic activity is justified by the fact that the mitoses, with rare exceptions, were located in the basement layer.

The increase in body weight which occurred in the thyroxine-treated rats might conceivably produce a stretching of the epidermis. Consequently, even though the number of mitoses over the whole skin surface of the body might remain unchanged, a decreased number of mitoses per unit length of basement membrane would be observed. An attempt was therefore made to calculate the total number of mitoses in the epidermis over the

TABLE 3. EFFECT OF TESTOSTERONE AND THYROXINE ON THE MITOTIC ACTIVITY OF THE EPIDERMIS IN CASTRATED-THYROIDECTOMIZED RATS

	Control	Testosterone	Thyroxine	Testosterone and thyroxine
Mean number of mitoses per cm. of basement membrane (metaphases and anaphases only)	67	122	32	45
Mean number of mitosis per body surface ($\times 10^6$)	38.6	70.1	24.8	35.7
Ratios of:				
Thickness of epidermis to number of mitoses	0.76	0.85	0.97	1.76
Thickness of Malpighian layers to number of mitoses	0.35	0.24	0.51	0.56
Thickness of cornified layers to number of mitoses	0.41	0.62	0.45	1.20

Statistical Analysis: P values

	Control vs. testosterone	Control vs. thyroxine	Control vs. testosterone and thyroxine	Testosterone vs. thyroxine	Testosterone vs. testosterone and thyroxine	Thyroxine vs. testosterone and thyroxine
Mean no. of mitoses per cm. of basement membrane	0.02	0.10	0.30	0.001	0.001	0.40

whole body surface. For this purpose, two assumptions were made: firstly, that the degree of mitotic activity was the same everywhere in the epidermis, and secondly, that the number of mitoses counted per cm. of basement membrane was equal to the number present in an area of epidermis of 0.0005 cm.^{2} (the thickness of the sections being 0.0005 cm.). The total surface area of the rat in cm.^{2} was then obtained by using Meeh's formula, $11.2 \times [\text{body weight (gm.)}]^{2/3}$. By multiplying the number of mitoses per cm. of basement membrane by surface area/0.0005, the number of mitoses in the whole epidermis was obtained.

Standard errors of the measurements of epidermal thickness and mitotic rate were determined by analysis of variance, and significant differences evaluated by the "t" test. Probability values (P) less than 0.05 were considered significant (Tables 2 and 3).

Since the number of mitoses was found to increase as epidermal thickness increased, the regression coefficient between them was calculated. The number of mitoses per cm. (x) was taken as the independent variable, and the thickness of the whole epidermis (y) as the dependent variable. These were plotted (Figure 5); the regression line was calculated, and its significance evaluated by the "t" test.

RESULTS

Microscopic examination of the epidermis indicated that testosterone alone increased the size of Malpighian and cornified layers, as may be seen by comparing untreated control (Fig. 1) with testosterone-treated animals (Fig. 2), while thyroxine alone decreased the size of these layers (Fig. 3).

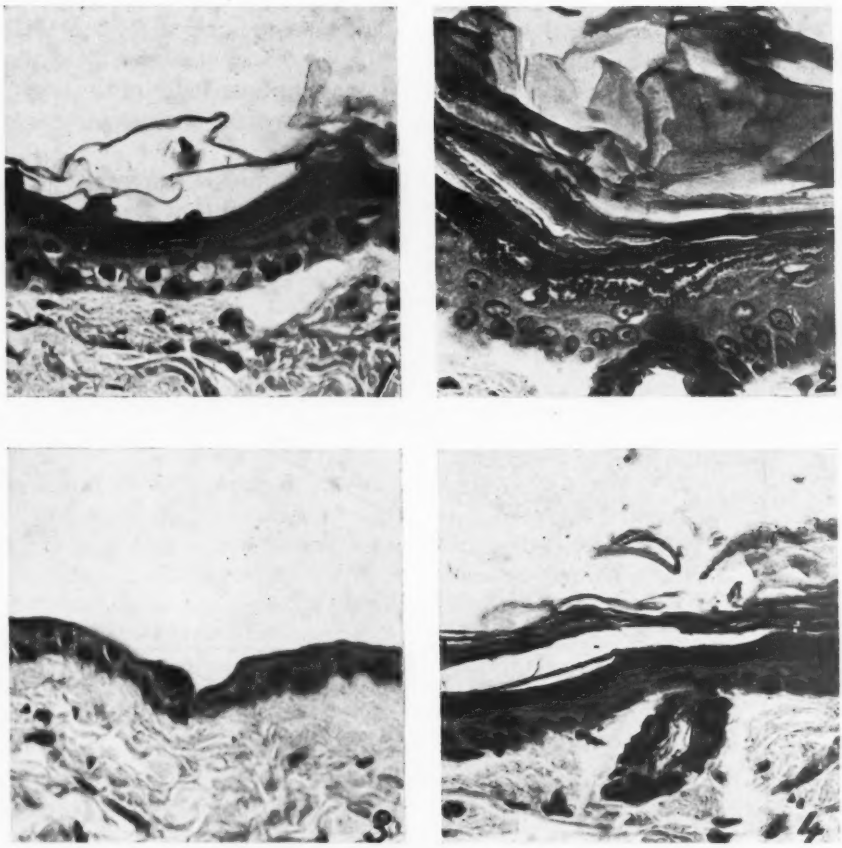

PLATE I. Epidermis from animals of the first experiment.
Orth fixation. H & E stain. ×296.

EXPLANATION OF FIGURES

FIG. 1. Control castrated-thyroidectomized rat. Note the thickness of the Malpighian and cornified layers, as well as the presence of granules in the stratum granulosum.

FIG. 2. Castrated-thyroidectomized rat treated with testosterone. Malpighian and cornified layers are greatly increased in size as is also the number of granules.

FIG. 3. Castrated-thyroidectomized rat treated with thyroxine. Malpighian and cornified layers are greatly decreased in size, and the granules are almost absent.

FIG. 4. Castrated-thyroidectomized rat treated with testosterone and thyroxine. The thickness of the Malpighian and cornified layers is intermediate between that of rats treated with testosterone and that of rats treated with thyroxine, and approximated that of the control animals.

Both hormones given together produced an intermediate effect (Fig. 4).

The stratum germinativum and the stratum granulosum of the Malpighian layer were similarly affected by the various treatments. Thus, the quantity of granules—which presumably was simply an index of the thickness of the stratum granulosum—was clearly increased after testosterone and decreased after thyroxine.

When the thickness of the Malpighian and cornified layers was subjectively assessed as numbers of "+," it could be shown that the effects of

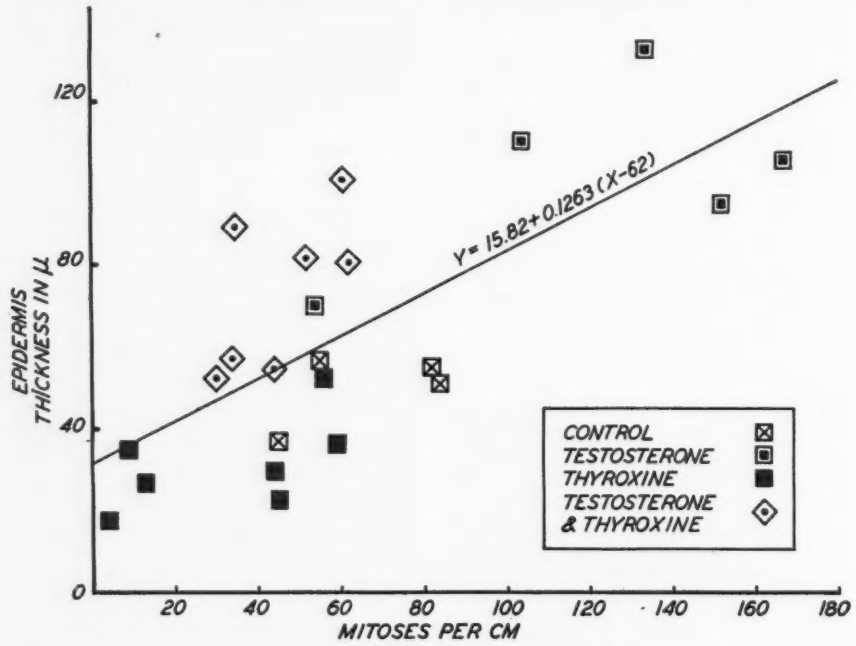

FIG. 5. The regression of the number of mitoses per cm. of basement membrane over the epidermal thickness (in μ) in the rats of the four groups in the first experiment.

testosterone and thyroxine were similar in the three experiments under study (Table 1). The normal, sham-operated controls usually had an epidermal thickness intermediate between those of testosterone and thyroxine-treated animals.

The histometric studies carried out in the first experiment revealed essentially similar results (Table 2). Thus, testosterone significantly increased epidermal thickness, while thyroxine decreased it, the latter effect being on the verge of significance. When the effect on the individual layers of the epidermis was examined, testosterone produced a significant en-

largement of the corneum, and thyroxine caused a significant thinning of the Malpighian layer. When the effect of testosterone alone was compared to that of thyroxine alone, a highly significant difference in the thickness of both layers was observed. When testosterone and thyroxine were administered together, the picture was an intermediate one, differing significantly from that of the testosterone group and very significantly from that of the thyroxine group.

The number of mitoses per unit length of basement membrane (Table 3) was significantly increased by testosterone. Thyroxine decreased the number, but the effect was on the verge of significance (as was also its effect on epidermal thickness, Table 2). Furthermore, the regression of mitotic number over epidermal thickness was highly significant ($P < 0.001$). Hence, epidermal thickness varied as mitotic activity.

The total number of mitoses of the body surface (Table 3) varied like the number of mitoses per centimeter of basement membrane. This result indicated that the effects of the hormone on the skin were direct and not secondary to a variation of its surface area.

An incidental observation was made in the second experiment in which the animals were shaved on one side of the body a week before the end of the experiment. Subjective inspection indicated that thyroxine accelerated the growth of hair, testosterone seemed to inhibit it, and both gave an intermediate effect. Histological examination of the number of follicles showed the same trend. Thus, the influence of the two hormones on hair growth seemed to be the reverse of their effect on the epidermal thickness.

DISCUSSION

The life history of the epidermal cell of the foot pad of the rat has recently been investigated (Storey and Leblond, 1950; and unpublished results). The cell arises by mitosis in the deeper part of the stratum germinativum, remains in this layer for about 16.9 days, spends the next 2.2 days in the stratum granulosum where it becomes flat and fills up with granules, and remains for a month as a cornified cell, after which it desquamates. Preliminary results indicated that the epidermal cells of the back and sides go through a similar, though shorter, life cycle.

All other things being equal, if the life span of the epidermal cell were to be increased or decreased, the thickness of the epidermis would automatically increase or decrease. Similarly, if the mitotic rate were increased or decreased, the number of cells present would increase or decrease, and so would the thickness of the epidermis.

In the present work, it was found that testosterone increased, and thyroxine decreased the thickness of both layers of the epidermis, while the joint action of the two hormones produced an intermediate condition,

somewhat comparable to that in normal skin. It was, therefore, of interest to establish whether these effects were due to an influence on the mitotic rate or on the life span of the epidermal cells or on both.

Counts of the number of metaphases and anaphases per cm. of basement membrane revealed that the number of mitoses varied in the same direction as the epidermal thickness (Fig. 5). Indeed, the regression of the former over the latter was highly significant. It was, therefore, concluded that changes in mitotic activity were the main cause of the observed changes in epidermal thickness.

The mechanism by which testosterone increased the mitotic rate and hence the epidermal thickness was not known, but a recent paper by Bullough and Eisa (1950) suggested a possible mechanism for thyroxine's action. These authors showed that the diurnal variation in the number of epidermal mitoses was strictly parallel to the diurnal variation of the glycogen content of the epidermis and also of the liver. This indicates that the energy required for mitosis is supplied by glycogen. Now, thyroxine treatment was shown to decrease the glycogen level of liver (Defauw, 1930; Fieschi, 1933; Coggeshall and Greene, 1933; Sternheimer, 1939) and heart (McDonald, Boyle and de Groat, 1938; Moses, 1944). Should a similar lowering of the glycogen level be produced by thyroxine in the epidermis, it would account for the decrease in mitotic activity.

However, if changes in mitotic rate were the sole factor involved, the thickness of the epidermis should be directly proportional to the number of mitoses. This was not the case, since the ratio of the mean epidermal thickness to the number of mitoses per cm. of basement membrane varied in the different groups (Table 3). An attempt to explain these results required a separate examination of the Malpighian and cornified layers.

The ratio of the Malpighian layer thickness to the number of mitoses (Table 3) was higher in the two thyroxine-treated groups than in the two others. A possible explanation of this fact was that the average size of the cells was greater in the thyroxine-treated groups; but examination of the slides did not reveal any clearcut difference in cell size. A more likely possibility was that, in the case of thyroxine treatment, the cell spent more time in the Malpighian layer.

The situation in the cornified layer was quite different, for the ratios of its thickness to its number of mitoses was greater in the two testosterone-treated than in the other groups. This finding suggested that testosterone may have increased the time a given cell spends in the cornified layer. Possibly, desquamation is inhibited by this hormone.

Finally, it may be noted that in experiments 2 and 3 the epidermis of intact rats showed a thickness intermediate between that of the testosterone- and of the thyroxine-treated animals, and one which was somewhat

comparable to that of the animals treated with both hormones (Table 1). Since testosterone is normally a hormone of the rat testis (Nelson, 1937; Moore, 1939) and thyroxine, a hormone of the rat thyroid (Leblond and Gross, 1948; Gross and Leblond, 1951), it is likely that, under physiological conditions, the epidermis of the male rat is chiefly maintained by a balance between these two hormones.

SUMMARY

The influence of testosterone and thyroxine on the histology of the epidermis was investigated in castrated-thyroidectomized male rats. They were injected subcutaneously for 6 to 7 weeks with daily doses of 0.5 mg. of testosterone, or 5–8 micrograms of DL-sodium thyroxine, or both. Subjective (Table 1) and objective (Tables 2 and 3) investigations of the epidermis of the back and sides were made.

Testosterone alone increases, and thyroxine alone decreases epidermal thickness, while combined treatment with both hormones results in an intermediate thickness (Figs. 1–4; Tables 1 and 2).

Similarly, testosterone alone increases, and thyroxine alone decreases the number of epidermal mitoses, while combined treatment with both hormones results in an intermediate number of mitoses (Table 3). The regression of the number of mitoses over the epidermal thickness is highly significant ($P < 0.001$); and, therefore, the effect of these hormones on the thickness of the epidermis is mainly mediated by way of their action on cell production. However, the data also indicate the possibility of an effect of these hormones on the life span of the epidermal cell.

It is concluded that, in addition to the well-recognized androgenic function of testosterone and metabolic function of thyroxine, these hormones may jointly act on a variety of end organs, either additively as on the hypophysis, synergistically as on the heart, kidney, and submaxillary gland, or antagonistically as on the epidermis.

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EFFECT OF HYPOPHYSECTOMY AT THE BEGINNING OF PROESTRUS ON MATURING FOLLICLES IN THE OVARY OF THE RAT[1]

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DURING the past twenty years a large amount of information has accumulated concerning the relationship between the gonadotrophic hormones and various aspects of ovarian development and function, but there are still many questions which have not been fully answered.

The increasing use of gonadotrophic hormone preparations to induce follicular maturation and ovulation in the human being and in domestic animals has reemphasized the need for more precise qualitiative and quantitative data concerning the hormonal factors necessary for the maintenance and development of maturing follicles just prior to ovulation.

Hertz and Meyer (1946) have shown that removal of the pituitary gland in the rat at the beginning of proestrus will prevent ovulation from taking place during the subsequent 46 hour period. From this work it was concluded that a hypophyseal hormone (LH) is required for ovulation in the rat and the necessary quantity is secreted subsequent to early proestrus. However, it was not possible to determine from this work whether the continued presence of the pituitary gland during proestrus is necessary for the maintenance of the maturing follicles in a condition where they will ovulate in response to an injection of LH.

The work reported here was undertaken to study the effect of hypophysectomy at the beginning of proestrus on the most mature follicles in the ovary of the rat during that period. The problem was approached first by studying the effect of hypophysectomy at the beginning of proestrus on the ability of mature follicles to repond to a hormonal ovulating stimulus, and secondly by studying the atretic changes which occur in these follicles following removal of the pituitary gland. A preliminary report of this study has been made by Meyer, Talbert, and McShan (1947).

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PROCEDURES

Ninety-eight young adult female rats of the Holtzman and Sprague-Dawley strains were used in a study of the effect of varying intervals of gonadotrophic hormone deficiency during proestrus on the ability of the most mature set of follicles to ovulate in response to an intravenous injection of gonadotrophin. Eighty-six of these rats were injected with a human chorionic gonadotrophin preparation while the remaining twelve were injected with an unfractionated sheep anterior pituitary preparation.

All the animals were hypophysectomized within three hours after the beginning of proestrus and injected with chorionic or pituitary gonadotrophin after an interval of 0 to 24 hours following the operation.

The beginning of proestrus was determined by examination of the vaginal smear pattern of all animals daily before noon. Those rats which were expected to begin proestrus on a given day and were not already in proestrus at the time of the first smear, were isolated and smeared every three hours until a typical proestrus type smear was obtained. A typical proestrus smear was designated as one which contained at least 90% nucleated epithelial cells with no leucocytes.

The chorionic gonadotrophin was prepared essentially by the method of Gurin, Bachman, and Wilson (1939). A fifty or one hundred microgram dose of the dry powder dissolved in an alkaline saline solution at a concentration of 100 micrograms per cc. was injected into the tail vein or heart of each animal.

The pituitary gonadotrophin was prepared in this laboratory by the method described by McShan and Meyer (1943). One hundred milligrams equivalent of fresh pituitary tissue dissolved in normal saline at a concentration of 200 milligrams equivalent per cc. was used as the ovulating dose.

Human chorionic gonadotrophin was used as the ovulating hormone for most of the experimental animals because this substance is known to be almost entirely luteinizing in its action and will not induce maturation of follicles in hypophysectomized animals. Since there is no definite proof that this hormone preparation can induce ovulation in the complete absence of other gonadotrophic hormones it was necessary to inject a small number of animals with a pituitary gonadotrophic preparation which is known to be capable of inducing ovulation.

Autopsies were carried out thirty to sixty hours after the administration of the gonadotrophin. The sella turcica of each rat was examined with magnifying spectacles to determine if the entire pituitary had been removed. The ovaries and oviducts were removed and fixed in FAA (one part glacial acetic acid, one part formalin, and three parts 95 per cent alcohol) or Helly's fixative.

Serial sections of one ovary and oviduct from each rat were cut at eight micra and stained with hematoxylin and eosin. The number of new corpora lutea, mature unruptured follicles, and ova in the oviduct were determined. The presence of eggs in the oviduct was used as evidence that ovulation had taken place.

Thirty-three animals were used in a study of the effect of hypophysectomy at the beginning of proestrus on atresia of maturing follicles. Twenty-one of these animals were hypophysectomized at the beginning of proestrus and autopsied at intervals of four to fifty hours following the operation. The remaining rats were untreated and autopsied at intervals corresponding to the operated animals until ovulation had taken place.

One ovary and oviduct from each rat was fixed in Helly's solution and stained either with hematoxylin and eosin or by the Feulgen nucleal method as described by Cowdry (1948).

Two criteria were used as indicators of atresia of the most mature set of follicles: (1)

pyknotic nuclei in the granulosa cells and (2) degenerative changes in the ova in these follicles.

Degenerative changes in the ova were determined by the absence of the nucleus, nucleoli, and chromatin material.

OBSERVATIONS

The effect of different intervals between hypophysectomy and the injection of a gonadotrophic hormone preparation on the ovulating ability of maturing follicles is shown in Table 1.

These data show that an interval of 0 to 6 hours between the removal of the pituitary gland and the injection of chorionic gonadotrophin does

TABLE 1. EFFECT OF INTERVAL BETWEEN HYPOPHYSECTOMY IN EARLY PROESTRUS AND INJECTION OF CHORIONIC OR PITUITARY GONADOTROPIN ON THE PERCENTAGE OF RATS OVULATING

Interval "hypophysectomy" to injection	No. of rats	No. ovulated	% ovulated
	Chorionic Gonadotrophin		
0 hours	6	6	100
2 hours	6	5	83
4 hours	11	10	91
6 hours	7	6	86
8 hours	23	18	78
10 hours	14	6	44
12 hours	8	1	13
19 hours	6	0	0
24 hours	5	0	0
	Pituitary Gonadotrophin		
6 to 8 hours	4	3	75
12 hours	8	1	13

not significantly influence the percentage of mature follicles which will ovulate following the injection.

Extension of the interval between the operation and the injection to 8 to 12 hours results in a rapid decrease in the percentage of mature follicles which will ovulate.

When the interval is extended to 19 hours or more none of the mature follicles will ovulate.

The results obtained from the animals injected with pituitary gonadotrophin are also shown in Table 1. From these data it can be seen that three of four animals injected 6 to 8 hours after hypophysectomy ovulated while only one of eight injected 12 hours following the operation responded.

In addition to the observations summarized in Table 1 it was found that nearly all of the large follicles from rats which were injected by 12 hours following hypophysectomy had become luteinized whether they had ovulated or not, while the ovaries of rats injected 19 and 24 hours following the operation rarely showed this change. Figures 1 and 2 show large follicles which are typical of each condition.

Our studies of atresia have shown that the most mature set of follicles in the ovary of the rat rarely contain pyknotic granulosa cell nuclei at any time during proestrus. The study of similar stages in rats which have been hypophysectomized at the beginning of proestrus showed that pyknotic cells are very rare 12 hours after hypophysectomy but are common by 19 hours following the operation.

Degenerative changes were not noted in the ova 19 hours following hypophysectomy but they were obviously degenerating after 48 hours. Such ova are also quite often flattened against the wall of the antrum, and in a few cases maturation changes can be found.

DISCUSSION

In our opinion there are two possible factors which may have been responsible for failure of ovulation to occur beyond 8 to 12 hours following hypophysectomy in response to an injection of chorionic gonadotrophin.

The first possibility is that some change had occurred in these large follicles during the interval following hypophysectomy which makes them incapable of responding to a hormonal ovulating stimulus.

The second possibility is that chorionic gonadotrophin alone is not capable of inducing ovulation but acts synergistically with pituitary gonadotrophin still circulating after hypophysectomy. In this case the interval between the removal of the pituitary and the injection of chorionic gonadotrophin was so long that the pituitary gonadotrophin remaining in the blood had dropped to a level below that necessary to synergize with the injected hormone to induce ovulation. The latter possibility is supported by the fact that there is no satisfactory evidence that human chorionic gonadotrophin is capable of inducing ovulation in the complete absence of other gonadotrophins.

Hinsey and Markee (1933) reported that ovulation, using chorionic gonadotrophin as the ovulating hormone, could be induced consistently in rabbits in which the midbrain and the pituitary had been removed.

Description of Figures

Fig. 1. Follicle from rat injected with chorionic gonadotrophin twenty-four hours after hypophysectomy at the beginning of proestrus. Note the absence of luteinization.

Fig. 2. Follicle from rat injected with chorionic gonadotrophin ten hours after hypophysectomy at the beginning of proestrus. A marked degree of luteinization is evident.

Fig. 3. Portion of follicle of rat killed nineteen hours following hypophysectomy at the beginning of proestrus demonstrating marked pyknosis of the granulosa cells. The few dark pyknotic nuclei can be seen deep in the granulosa as well as on the surface.

Fig. 4. Portion of follicle of rat killed fifty hours following hypophysectomy at the beginning of proestrus. Dark staining pyknotic nuclei are evident throughout the granulosa layer while the ovum has become flattened against the wall of the follicle.

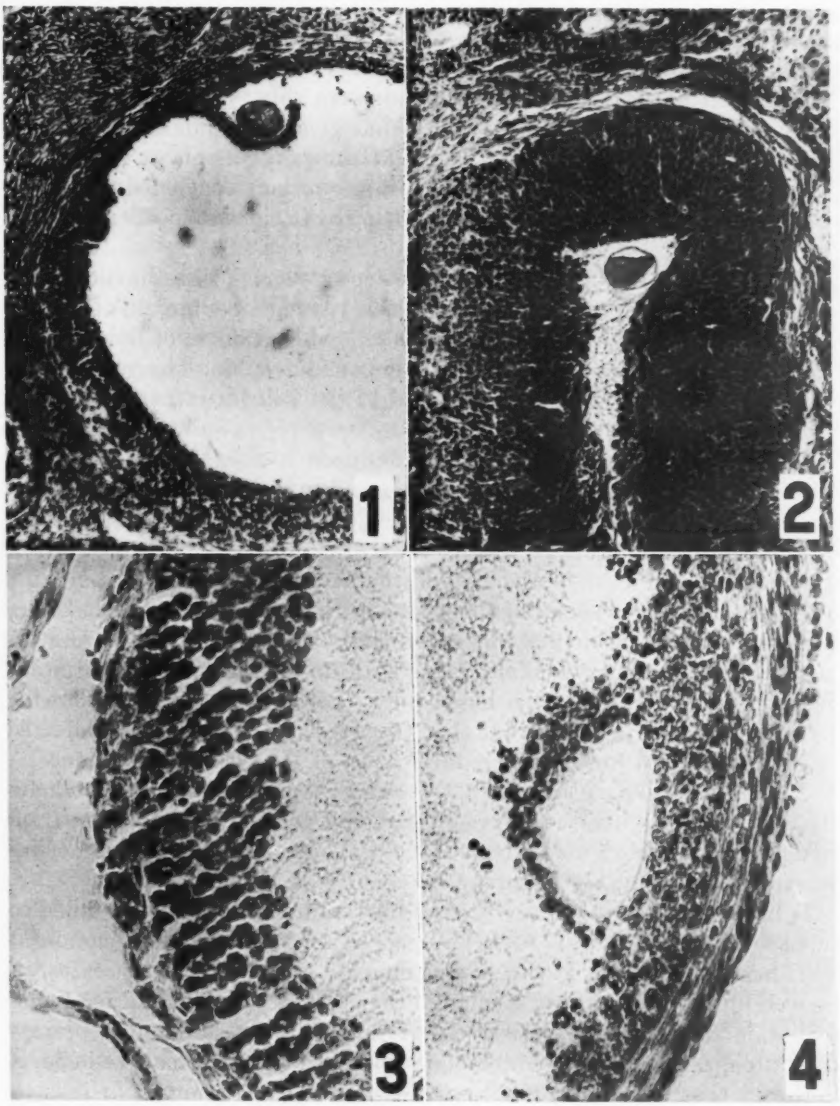

PLATE 1

Ovulation could not be obtained, however, if the injection was delayed more than two hours after the operation. These results were interpreted as indicating that the injected chorionic gonadotrophin had to act with the residual pituitary gonadotrophin in the blood to induce ovulation. White and Leonard (1933), on the other hand, were able to induce ovulation in hypophysectomized rabbits with chorionic gonadotrophin and found that only a slightly higher dose was required than was necessary to induce ovulation in intact animals. On this basis these workers concluded that chorionic gonadotrophin was capable of inducing ovulation independently of the animal's own pituitary gland.

We believe that our results are in better agreement with the interpretation of White and Leonard because pituitary gonadotrophin did not induce ovulation after a longer period of time following removal of the pituitary gland than was possible with chorionic gonadotrophin. This strongly indicates that some change has occurred in the follicles rather than in the hormonal stimulus.

Although this experiment was not designed to rule out the possibility that other hormones of the pituitary gland may be involved it appears most reasonable that the loss of the ability of maturing follicles to ovulate by 8 to 12 hours following hypophysectomy is due to a deficiency of one or both of the hormones of the gonadotrophic complex. In any event it is apparent that the presence of the pituitary gland is essential for the maintenance of maturing follicles in a normal condition during the proestrus period. Furthermore, when allowance is made for the fact that pituitary hormones are present in the blood for a short period of time following hypophysectomy it is apparent that these follicles are adversely affected within a very few hours by a reduced level of the pituitary hormones.

Since our results suggested that a change occurs in maturing follicles by 8 to 12 hours following hypophysectomy which prevents them from ovulating in response to an injection of gonadotrophin, it became of interest to investigate the nature of this change.

It has been pointed out above that the mature follicles, which failed to ovulate when the interval between hypophysectomy and the injection of gonadotrophin did not exceed twelve hours, were found, in most cases, to be well luteinized and very similar to the corpora lutea formed following ovulation. The possibility exists, therefore, that a large amount of premature luteinization had prevented ovulation from taking place. We believe, however, that this is not the most reasonable explanation for if it is to explain failure of ovulation in animals injected within twelve hours following hypophysectomy, it is necessary to postulate a different reason for failure of ovulation in the rats injected nineteen and twenty-four hours following the operation where luteinization did not occur. In our opinion

a more plausible explanation of the relationship of luteinization to failure of ovulation is that mature follicles in the ovaries of rats which have been hypophysectomized lose their ability to ovulate before they lose their ability to luteinize, but this property is also lost a few hours later.

A large number of studies have been made of the morphological changes associated with atresia of follicles of different degrees of maturity in several species of experimental animals but there has been little attempt to correlate these findings with physiological changes in these follicles (Pincus and Enzmann, 1937; Pliske, 1940; Vincent and Dornfeld, 1948).

Our studies of follicular atresia following hypophysectomy in the rat have shown that atretic changes cannot be detected in maturing follicles until 12 to 19 hours following the operation but follicles lose their ability to respond to an ovulatory stimulus between eight and twelve hours following hypophysectomy. On the basis of this observation it must be concluded that some physiological change has occurred in these follicles prior to the time that any histological change can be detected, at least by the methods employed in this study.

Although the nature of the physiological change which has prevented these follicles from ovulating is unknown Hisaw's (1947) suggestion that pyknosis of the granulosa cell nuclei may be precipitated by changes in the theca interna is worthy of investigation. This suggestion was based on the fact that the granulosa cells are dependent on the blood supply to the theca for their metabolic requirements and in addition the theca is believed to be the source of estrogen which may play a part in the growth and differentiation of the follicle.

SUMMARY

A study was made of the effect of hypophysectomy during proestrus on the ability of the most mature set of follicles to ovulate in response to an injection of human chorionic gonadotrophin and the relationship of this operation to atretic changes in these follicles.

The maturing follicles were found to lose their ability to ovulate by eight to twelve hours following removal of the pituitary gland, but histologically detectable atretic changes were not observed until nineteen hours following the operation. It was also observed that following hypophysectomy the maturing follicles lost their ability to ovulate several hours before they lost their ability to luteinze in response to an injected gonadotrophin.

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EFFECT OF HYPOPHYSECTOMY EARLY IN PROESTRUS ON THE VIABILITY OF OVA IN MATURING FOLLICLES[1]

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IN THE preceding paper (Talbert, Meyer, and McShan, 1951) a study was made of the effect of hypophysectomy at the beginning of proestrus on the ability of mature follicles to ovulate in response to an intravenous injection of chorionic or pituitary gonadotrophin. That investigation was extended to include a study of histological changes which occur in maturing follicles following removal of the pituitary gland.

It was determined in this study that mature follicles lose their ability to ovulate within 8 to 12 hours following hypophysectomy at the beginning of proestrus but no histological changes were detected until 19 hours following the operation. At this time a definite increase in pyknotic granulosa cell nuclei was noted but degenerative changes in the ova in these follicles were not apparent until many hours later.

Although the ova appeared by histological examination to be normal 6 to 8 hours following hypophysectomy it did not seem unreasonable that physiological changes may have occurred which would make them nonviable or incapable of normal development following fertilization. A study was therefore made of the effect of hypophysectomy at the beginning of proestrus on the viability and pre-implantation development of ova ovulated from mature follicles.

METHOD AND MATERIALS

Fifty-five three to five month old female rats of the Holtzman strain were used in this study. These rats were divided into five groups which were prepared as follows: group A, untreated animals; group B, normal animals injected with chorionic gonadotrophin at the beginning of proestrus; group C, animals hypophysectomized in early proestrus and injected with chorionic gonadotrophin immediately after the operation; group D, normal animals injected with chorionic gonadotrophin six to eight hours after

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the beginning of proestrus; group E, animals hypophysectomized at the beginning of proestrus and injected with chorionic gonadotrophin six to eight hours later.

The beginning of proestrus was determined for each animal by obtaining vaginal smears as was described in the preceding paper (Talbert *et al.*, 1952).

The rats in group A were inseminated naturally while those in the other four groups were artificially inseminated. This procedure was necessary since only a very small percentage of hypophysectomized female rats will mate. Artificial insemination was carried out 11 to 13 hours after the injection of gonadotrophin. This was calculated to be the approximate time of ovulation.

The technique used for artificial insemination was adapted from the method described by Blandau and Jordan (1941). The principle difference between our procedure and that of Blandau and Jordan was that all the spermatozoa were introduced into the right uterine horn instead of into both horns.

The animals were autopsied 13 to 113 hours following copulation or artificial insemination. The ova were flushed from the right oviduct with Locke's solution. If the interval between insemination and autopsy was long enough to permit eggs to pass through the entire oviduct, the right uterine horn was also flushed. The eggs were washed into a depression slide where they could be observed with a dissecting microscope. They were then transferred by means of a glass pipette to a standard slide, covered with a cover slip, which had a small amount of vaseline in each corner to prevent crushing of the eggs, and observed at a magnification of 450 with a dark contrast objective of a phase microscope.

OBSERVATIONS

In this study it was first necessary to establish criteria for distinguishing normal fertilized eggs from unfertilized and abnormal eggs. This problem was simplified considerably by previous studies of the early development of fertilized rat and mouse ova and the degeneration of unfertilized ova in the same species (Sobotta, 1895; Sobotta and Burckhard, 1911; Huber, 1915; and Charlton, 1917).

Austin and Smiles (1948) showed, by means of the phase microscope, that it was possible to observe the sperm tail within the zona pellucida of rat ova following fertilization. Our preliminary experiments have shown that the sperm tail can always be found within the zona pellucida of fertilized eggs through the two-cell stage of development, but following this stage the sperm have disappeared in some eggs which are apparently undergoing cleavage. After the two-cell stage normal development can be recognized best by the presence of cells of approximately equal size which are regular in outline. Unfertilized eggs were never found to divide equally beyond the initial cleavage and the fragments were usually irregular in shape.

Sixteen of the 87 fertilized eggs which were recovered contained more than one sperm tail within the zona pellucida but among these there was only one case in which it appeared certain that more than one sperm had penetrated the egg membrane. In addition to the absence of the sperm tail other reliable criteria were used to distinguish unfertilized from fer-

tilized eggs. These criteria are based on the changes which occur in ova following fertilization and the degenerative changes which occur in these ova if fertilization does not take place.

Unfertilized eggs never were found to have completed the second maturation division which has reached the metaphase stage at the time of ovulation whereas fertilized eggs contain a resting nucleus a few hours following fertilization.

Forty-eight hours following insemination fertilized eggs have nearly always divided into two or four cells.

Unfertilized eggs are quite variable in appearance by 48 hours following insemination. Some of these ova appear similar to unfertilized eggs a few hours following insemination but more often they contain two to eight small vesicles which give the appearance of multiple small nuclei each containing one or more rounded nucleolar-like structures. These round bodies have been shown by Charlton (1917) and Cowdry (1932) to consist of chromatin material and are thus not true nucleoli. In some cases unfertilized ova divide into two equal fragments but the presence of multiple nuclei can be used to distinguish these ova from normal two-cell stages. Figures 1 and 2 show, respectively, a normal two-cell stage and an unfertilized egg divided into two equal fragments. A few eggs have degenerated even further by this time. This degeneration is distinguished by fragmentation of the eggs into many irregular pieces. The larger fragments usually contain rounded clumps of chromatin material which may or may not be contained in nuclear vesicles. Final degeneration is indicated by dissolution of the cytoplasm which results in the complete disappearance of the eggs leaving the zona pellucida as an empty shell. Figure 3 illustrates an egg which has become nearly completely dissolved.

By 113 hours following insemination all fertilized eggs have developed to the morula (fig. 4) or blastocyst stage whereas unfertilized eggs have all frangmented into many small irregular pieces or have undergone varying degrees of dissolution.

The results shown in Table 1 indicate that under the conditions of this experiment we were not able to recover as high a percentage of normal eggs from any of the groups of animals which were artifically inseminated as was obtained in the animals which were naturally inseminated. In this respect we were unable to repeat the results obtained by Blandau and Jordan (1941) who claim to have obtained as high a percentage of fertilized eggs by artificial insemination as was possible with natural insemination.

It is also apparent that a period of six to eight hours between hypophysectomy at the beginning of proestrus and the injection of chorionic gonadotrophin, which was necessary to produce ovulation, did not reduce the

TABLE 1. EFFECT OF HYPOPHYSECTOMY AT THE BEGINNING OF PROESTRUS ON THE PERCENTAGE OF NORMAL EGGS RECOVERED FROM ONE OVIDUCT AND UTERINE HORN OF THE RAT

Group[1]	Total eggs recovered	Total normal eggs recovered	Percentage of normal eggs recovered
A	25	18	72
B	42	23	54
C	35	11	31
D	55	21	38
E	41	14	34

[1] Group A—Untreated rats; natural insemination.
Group B—Normal rats; injected beginning of proestrus; artificially inseminated.
Group C—Rats hypophysectomized at beginning of proestrus; injected immediately; artificially inseminated.
Group D—Normal rats; injected six to eight hours after beginning of proestrus; artificially inseminated.
Group E—Rats hypophysectomized at beginning of proestrus; injected six to eight hours later; artificially inseminated.

percentage of normal eggs which were recovered from the oviduct. This is shown by the results obtained in groups C and E.

There is only a slight difference between the two groups of artificially inseminated normal animals and the two groups of hypophysectomized animals. Therefore, there is little evidence to support the hypothesis that a deficiency of gonadotrophin while the eggs are in the oviduct and lumen of the uterus has any adverse effect on fertilization or on the early cleavage stages of development.

One other factor which might influence the viability of the eggs is premature ovulation. On the basis of unpublished data (Talbert, 1950) there is evidence that the animals in groups B and C ovulated six to eight hours prior to the time that ovulation would normally occur while the animals in groups D and E ovulated at the normal time. Since the ova from the animals which ovulated six to eight hours early were not different

DESCRIPTION OF FIGURES

FIG. 1. Two cell stage ovum from oviduct of rat in Group E autopsied 48 hours following artificial insemination. The sperm tail can be seen clearly lying across the two blastomeres.

FIG. 2. Unfertilized egg from rat in Group E which was autopsied 48 hours following artificial insemination. False nucleoli, some of which are in nuclear vesicles, can be seen scattered in the cytoplasm.

FIG. 3. Ovum near terminal stage of degeneration from rat in Group D autopsied 72 hours after artificial insemination. Notice that the cytoplasm has partially liquefied.

FIG. 4. Normal morula stage from rat in Group B autopsied 113 hours following artificial insemination. Notice that the cells are of approximately equal size which was never found in fragmented eggs.

Magnification of all photographs approximately 600×.

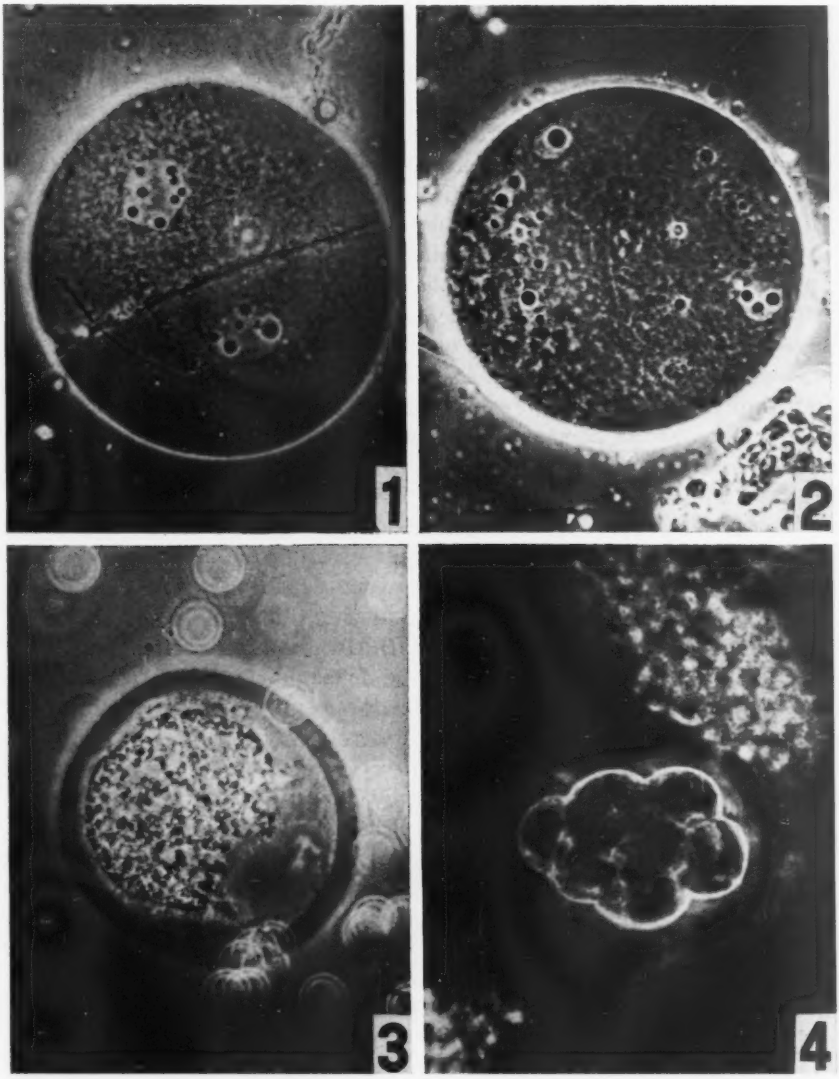

PLATE 1

from those ovulated at the normal time premature ovulation does not appear to be a factor in the viability of these eggs.

DISCUSSION

A few studies have been carried out to determine the effect of hypophysectomy or ovariectomy immediately following ovulation on the early development of mammalian eggs (Westman, Jorpes, and Widstrom, 1931; Firor, 1933; and Alden, 1942), but no work has been reported on the effect of hypophysectomy at the beginning of proestrus on the viability and early development of the mammalian egg.

There is no evidence which indicates that the gonadotrophic hormones have any direct influence on the eggs while they are in the lumen of the oviduct or uterus but there is some indication that the pituitary gland may indirectly affect these eggs by controlling the secretion of one or more of the ovarian hormones. This action of ovarian hormones has been demonstrated by Westmen *et al.* (1931) who found that ovariectomy or cauterization of the corpora lutea immediately after ovulation in the rabbit caused the ova to undergo degeneration. The degeneration of the ova was interpreted as being the result of failure of the ciliated tubal epithelium to change into a secretory type which occurs in the normal animal in response to progesterone stimulation. Firor (1933), on the other hand, found that hypophysectomy within three hours after copulation in the rabbit does not interfere with the development of the ova prior to implantation in the uterus. It would appear that sufficient quantity of progesterone and/or estrogen has been secreted by this time to maintain normal development of the ova.

In contrast to the results obtained by Westman *et al.* on the rabbit, Alden (1942) found that ovariectomy of the albino rat soon after ovulation does not interfere with the development of fertilized eggs prior to implantation. Therefore, it is not surprising that our results have shown that the pituitary gland is not essential for the maintenance of normal eggs while they are in the lumen of the oviduct and uterus. These studies have demonstrated that ovarian hormones are essential for the preimplantation development of ova in the rabbit but a similar requirement does not exist in the rat.

Our results have shown that an interval of six to eight hours between hypophysectomy at the beginning of proestrus and the injection of a gonadotrophin to induce ovulation does not result in a lower percentage of viable eggs than can be recovered from animals which are injected immediately after the operation. Since ovulation of the mature follicles cannot be induced regularly more than eight hours after hypophysectomy at the beginning of proestrus it is apparent that these follicles lose their

ability to ovulate before the viability of the ova has been affected. These results are in agreement with histological observations which have demonstrated that atresia occurs in the granulosa cells of large follicles before any degenerative changes are apparent in the ova in these follicles (Vincent and Dornfeld, 1948; Talbert *et al.*, 1952). Although our work does not rule out the possibility that gonadotrophic hormones may exert a direct action on the ova in mature follicles it appears far more likely that degeneration of these ova is dependent upon previous degeneration of the follicles in which they are contained.

Although our data failed to demonstrate that premature ovulation had any effect on the viability of the eggs which were recovered from the lumen of the oviduct and the uterus it should be kept in mind that this study included only the first four to five days after ovulation. The possibility remains, therefore, that abnormalities might become evident at a later stage of development. This has been shown to occur in the fowl by Neher and Fraps (1946). They induced ovulation 24 hours in advance of the normal time and found there was no apparent effect on the early stages of development but the percentage of eggs which hatched was reduced. This indicates that in this species prematurely ovulated eggs are not normal although the abnormality does not become apparent morphologically until late in development. It is obvious that a similar effect could result from gonadotrophic hormone deficiency prior to ovulation. This hypothesis may be tested only by a study of later stages of development which would require methods that are different from the ones employed in this study.

SUMMARY

An investigation was made of the effect of removal of the pituitary gland at the beginning of proestrus on the viability of eggs in maturing follicles of the rat. It was shown that the absence of the pituitary gland for a period of six to eight hours during proestrus does not prevent these ova from undergoing normal early development following artificially induced ovulation. It was also shown that the pituitary gland hormones are not essential, directly or indirectly, for the development of fertilized ova while they are in the lumen of the oviduct or uterus.

Eggs which were ovulated six to eight hours prematurely by an intravenous injection of chorionic gonadotrophin were capable of normal early development.

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THE EFFECT OF FRACTURES UPON URINARY ELECTROLYTES IN NON-ADRENALECTOMIZED RATS AND IN ADRENALECTOMIZED RATS TREATED WITH ADRENAL CORTEX EXTRACT

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ONE of the characteristic metabolic responses of man and of laboratory animals to injury is a temporary decrease in the urinary excretion of sodium and chloride (see Selye, 1950, for review). Since certain of the hormones of the adrenal cortex cause the retention of these ions and since the secretory activity of the adrenal cortices is increased during any type of stress, we have tested the hypothesis that the retention of sodium and chloride following fractures in the rat is caused by an increase in the secretion of cortical hormones. Adrenalectomized rats treated with a constant intake of adrenal cortex extract were found to show a striking temporary reduction in the urinary sodium and chloride following multiple fractures of the back legs just as did non-adrenalectomized rats.

METHODS

Male rats of the Sprague-Dawley strain were adapted to the force feeding of a medium carbohydrate diet (table 1) which was administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The technique of force feeding and the diets used were modifications of those described by Reinecke, Ball and Samuels (1939). The rats were brought to a full feeding on the 5th day. After the rats were adapted to the diet, 6 animals were adrenalectomized and 6 animals were subjected to a sham operation in which the adrenals were exposed but were not damaged. Asepsis was successfully maintained in these operations. The adrenalectomized rats were treated with 4 cc. of adrenal cortex extract per rat per day. The extract represented 40 grams of beef adrenal glands per cc., it contained 0.9 per cent NaCl and was free from alcohol. It was injected subcutaneously in divided doses each morning and afternoon. The sham operated rats were given control injections of 0.9 per cent NaCl solution.

Fractures were made under ether anesthesia. Care was taken to collect the urine excreted at the time the ether was administered.

The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78 degrees F. Twenty-four-hour samples of urine were collected at the same hour each day and preserved with toluene and citric acid (1 gm. per sample) to insure the acidity of the urine for nitrogen analyses. Urinary non-protein nitrogen was determined by the micro-Kjeldahl procedure as follows: Proteins were precipitated as

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TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	g.	Mg.
Cellu flour (Chicago Dietetic Supply)	120	
Osborne & Mendel Salt mixture	40	
Dried yeast (Pabst)	100	
Wheat germ oil	10	
Cod liver oil	10	100
Vit. K (2-methyl-1,4-naphthoquinone)		100
Mazola oil	200	
Casein (Labco)	160	
Starch	200	
Dextrin	190	
Sucrose	200	
Water to make total of	2000 cc.	

the salts of tungstic acid by the Folin-Wu procedure. The organic matter was oxidized by sulfuric acid and hydrogen peroxide. The ammonia was distilled off into a standard acid solution and titrated with standard base. Urinary chloride was determined by the modified Volhard-Harvey titration as described by Peters and Van Slyke (1932) and urinary sodium and potassium were determined by flame photometry.

EXPERIMENTS AND RESULTS

Two weeks following operation, 6 adrenalectomized rats which were treated with ACE and 6 sham-operated rats given control injections of saline were subjected to fractures of the tibia, knee joint and femur of each back leg. The experiments were terminated 10 days following the fractures. The data are summarized in Figure 1.

All of the animals gained weight up to the time of injury. All of the rats showed a sharp gain in weight immediately following the fractures, but within 48 hours the animals began to lose weight for periods of 4 or 5 days whereupon weight gains were renewed.

All of the animals were in positive nitrogen balance up to the time of injury. Following the fractures, the adrenalectomized rats excreted less nitrogen during the first 24 hours, but thereafter showed a marked rise in the level of urinary nitrogen which paralleled that of the non-adrenalectomized series. By the end of 7 days, a positive nitrogen balance was re-established.

The changes in excretion of potassium following fractures paralleled the changes in nitrogen excretion in each series of animals. The adrenalectomized rats, but not the controls, showed a fall in the level of urinary potassium during the first 24 hours following injury.

All of the rats showed a striking retention of sodium and chloride during the first 24 hours following injury. By 72 hours, there was a compensatory increase in the excretion of each ion which coincided with a disappearance of edema from the area of injury and a rise in urine volume. Subsequently all of the animals remained in sodium and chloride balance.

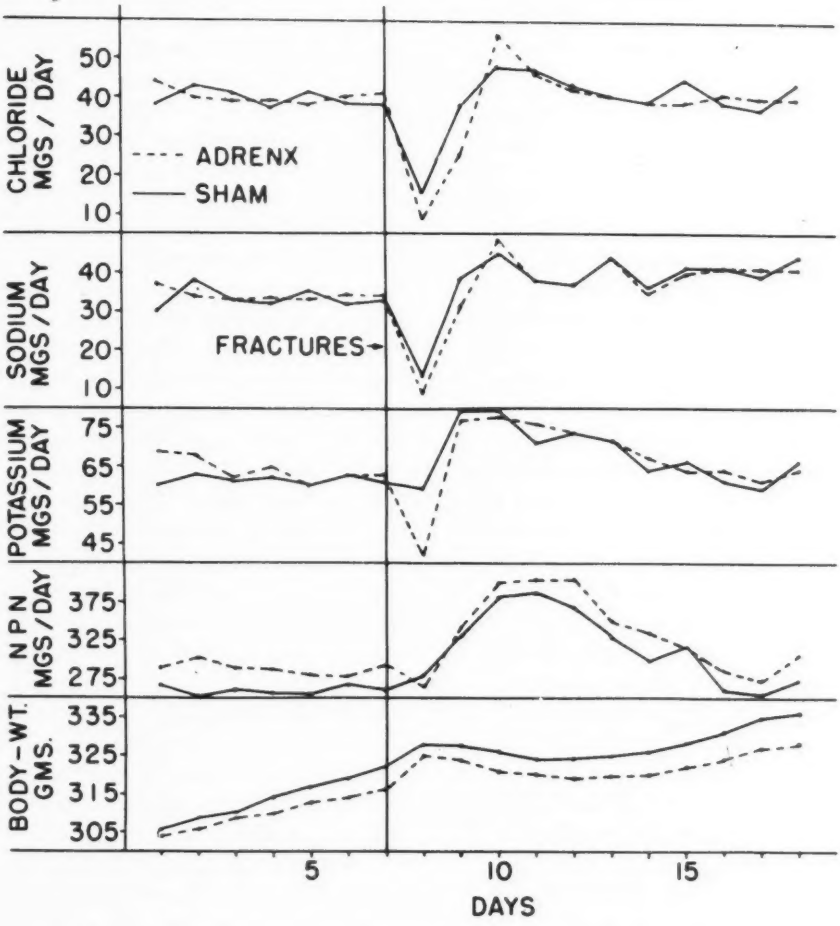

FIG. 1. Metabolic responses to fratures in non-adrenalectomized and adrenalectomized force fed rats. The adrenalectomized rats received 4 cc. of ACE per rat per day throughout the experiment. Averages for six rats per group.

DISCUSSION

The adrenal cortex is commonly described as a regulator of electrolyte metabolism. According to this concept, the secretory activity of the adrenal cortex is either directly or indirectly sensitive to the concentration of inorganic ions in the body fluids and responds to abnormal concentrations of these ions by secreting more or less of certain steroids so that homeostasis is re-established. It is true that electrolyte balance is affected

by both adrenal cortical insufficiency and excess, and it has been shown that the morphology of the adrenal cortex can be affected by diets deficient in sodium chloride (Deane, Shaw and Greep, 1948), but these observations do not prove that the gland is a regulator.

The most significant support for the hypothesis that the adrenal cortex is a regulator of electrolyte balance has come from the studies of Conn and his associates (1946; 1949a; 1949b; 1950), on the role of the adrenal cortex in the acclimatization of man to humid heat. It was shown that adaptation to heat includes a marked reduction in the amounts of sodium chloride excreted in sweat, and it was postulated that this is due to activation of the adrenal cortices to secrete increased amounts of salt-retaining steroids during the exposure to humid heat. The supporting evidence is as follows: The administration of either adrenal cortex extracts or 11-desoxycorticosterone acetate reduces the NaCl content of sweat; activation of the adrenal cortices by the administration of adrenocorticotrophic hormone reduces the NaCl content of sweat; the sweat of adrenally insufficient patients has a higher than normal amount of NaCl whereas that of patients with hypercorticalism is lower than normal; acclimatization of human subjects to humid heat is accompanied by a rise in excretion of 17-ketosteroids thereby indicating that the adrenal cortex has been activated; and finally, when the acclimatized subject is treated with 11-desoxycorticosterone acetate, the withdrawal of this steroid is followed by a temporary loss of acclimatization which suggests that the secretory activity of the adrenal cortices have been suppressed. These observations do indeed offer strong support for the hypothesis that the concentration of NaCl in sweat is governed by the secretory activity of the adrenal cortex, but the evidence is circumstantial and, in our opinion, does not represent proof. Quite as strong arguments can be made for the hypothesis that the negative nitrogen balance which follows injury is due to the increase in the secretory activity of the adrenal cortices. It is known that hypercorticalism does cause a rise in urinary nitrogen, that the adrenal cortices are activated by injury, and that this response to injury does not occur in the adrenally insufficient animal. However, it has been shown earlier (Ingle, Ward and Kuizenga, 1947) and confirmed in the present study that adrenalectomized animals given a uniform intake of adrenal cortex extract still show a normal metabolic response to injury. It is evident that the metabolic response was not caused by the adrenal cortex although the presence of cortical hormone is essential to support the overt manifestation of response.

The present experiments also show that the urinary retention of sodium and chloride which occurs following fractures is independent of any change in the secretory activity of the adrenal cortices. Selye (1938) has previously

shown that the characteristic triphasic response of blood chlorides to injury occurs in the untreated adrenalectomized rat as well as in the non-adrenalectomized rat.

Although the present experiment provides a basis for challenging the hypothesis that the adrenal cortex is a true regulator of electrolyte metabolism, these data do not disprove it. No one has claimed that the adrenal cortex is the only mechanism regulating electrolyte metabolism. There are innumerable naturally occurring and experimental situations in which electrolyte balance is affected or in which the distribution of ions across membrane barriers is modified. The single response which we have selected for study may not represent a homeostatic adjustment. The retention of sodium and chloride ions in the water which accumulates in the area of injury may represent a physical phenomenon only, although it seems probable that there is a concomitant adaptive response in the kidney which permits an almost complete reabsorption of sodium and chloride by the tubules. Although there is some reduction of urine volume following injury, the kidney does not retain nitrogen and potassium to the extent that it retains sodium and chloride.

The question as to whether the adrenal cortex is a regulator of the metabolic processes which its hormones affect has been discussed at greater length elsewhere (Ingle, 1950, 1951). We have shown that a few metabolic responses thought to be regulated by the adrenal cortex can occur in its absence although the presence of the cortical hormones is required to support the response (Ingle, 1943; Ingle, Ward and Kuizenga, 1947; Ingle and Baker, In Press). Many other functions thought to be controlled primarily by the adrenal cortex remain to be examined. We suggest that the procedure followed in the present study is useful in elucidating the relationship of adrenal cortex function to a biologic response, namely, the comparison of the response of non-adrenalectomized animals with that of adrenalectomized animals on a fixed intake of cortical hormones. A fixed intake of food and uniformity of other experimental conditions is also required.

SUMMARY

This study tested the hypothesis that the retention of urinary sodium and chloride following fractures is due to an increased secretion of adrenal steroids. Male rats of 300 gms. initial weight were given a uniform intake of a medium carbohydrate diet by stomach tube. Six rats were subjected to sham operations, and an equal number were adrenalectomized and given a uniform intake of beef adrenal extract (4 cc. per rat per day) during the remainder of the experiment. Following a control period of 14 days, all of the rats were subjected to fractures of the tibia, femur and knee-joint of

both back legs. All of the rats showed a post-injury rise in urinary nitrogen and potassium and a sharp decrease in urinary sodium and chloride followed by a compensatory rebound and return to normal values. It was concluded that the observed responses to injury were not mediated by a change in the secretory activity of the adrenal cortices.

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LOCALIZATION OF RADIOACTIVITY AFTER ADMINISTRATION OF LABELED PROLACTIN PREPARATIONS TO THE FEMALE RAT

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PREVIOUS reports have indicated that anterior pituitary hormone preparations can be labeled with radioactive isotopes (Sonenberg, Keston and Money, 1950 and 1951). In these earlier studies, hormone preparations with adrenocorticotropic activity were labeled with traces of radioactive iodine, I^{131}. Preparations labeled in this manner were then used to investigate the localization of radioactivity after being administered to rats.

The technique of trace labeling proteins has been extended to other preparations containing pituitary hormones. This report presents the results of studies with prolactin preparations labeled with radioactive iodine.

MATERIALS AND METHODS

The techniques employed in trace labeling prolactin preparations were similar to those previously reported (Eisen and Keston, 1949; Sonenberg, Keston and Money, 1951).

The same prolactin preparation* was used throughout this study. In this preparation (Squibb #71713), there were 20 to 25 units of prolactin per mg. Adrenocorticotropic activity was also reported present as a contaminant in concentrations less than 0.03 International Unit per mg. No gonadotropic or thyrotropic activity was found on assay.

Fifteen milligrams of the hormone preparation were dissolved in a $NaHCO_3$-Na_2CO_3 buffer, pH 8.8. In another tube, approximately 25 micrograms of KI labeled with 20 millicuries of I^{131} were oxidized with nitrous acid, the excess acid being destroyed with ammonium sulfamate. This iodine solution was made alkaline with the bicarbonate-carbonate buffer and added to the dissolved hormone preparation. After mixing, the solution was allowed to remain at room temperature (22° C.) for one hour. The entire reaction mixture, except for a small aliquot for precipitation studies, was transferred to a Visking dialysis tube. The preparation was dialysed in the cold (4° C.) against four 17 liter changes of isotonic saline. All preparations were finally brought to a volume which

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would permit the injection of 1 ml. per animal. Radioiodinated bovine serum albumin (BSA)** was prepared in a similar fashion.

In order to determine the yield of iodinated protein, precipitation studies were done. To the retained aliquot of the reaction mixture was added 250 micrograms of KI, 0.1 ml. of 1N $Na_2S_2O_3$, and 5 milligrams of the prolactin preparation as carrier. The protein was precipitated by adding an equal volume of 10% trichloracetic acid. The precipitate was washed twice with 5% trichloracetic acid. The supernatants were pooled and the radioactivity in the precipitate and supernatant was determined. (About 25% of the radioactivity was found to be associated with the trichloracetic acid precipitate.)

TABLE 1. BIOLOGICAL ACTIVITY OF VARIOUS PROLACTIN PREPARATIONS. 1 TO 6 AND 1.6 TO 1 REPRESENT THE MOLECULAR RATIOS OF IODINE TO PROTEIN

Preparation	Pigeon weight, gms.	Crop sac weight, mgms.	Average crop sac weight, 100 gm. body wt.
Prolactin I^{131} #2 (1 to 6)	430 482	4000 4050	8.85
Prolactin I^{131} #3 (1.6 to 1)	540 420	5400 4000	9.76
Prolactin (Unlabeled)	485 470	4550 4150	9.10
Untreated Controls	452 382	2870 1920	5.69

For assay of biological activity, the pigeon crop sac technique (Riddle and Bates, 1939) was used. White Carneau pigeons, about 450 grams in weight, were injected over a three day period with either the labeled or unlabeled hormone preparation. The animals were sacrificed on the fourth day and the crops were removed, dissected and the crop sac glands weighed. Each preparation was assayed in two pigeons. As controls, two pigeons received no injection. Table 1 shows the assay of this material where the crop gland weights are expressed as the number of milligrams per 100 grams of body weight.

In the localization studies, female rats of the Sprague-Dawley strain were used. These animals ranged in weight from 50 to 300 grams. For each individual experiment, however, the rat weights were quite uniform. Comparisons have been made only between groups of animals of comparable weight. A number of lactating and pregnant rats were also used in localization studies. Approximately 200 rats were employed in these experiments. For the sake of brevity, the data from all these animals are not reported in this paper. The tables and figures record the results with 94 different animals with the results fairly representative of the various experiments.

Some mature rats were pretreated with chorionic gonadotropin in order to produce maximum luteal reaction. Each rat received 0.1 ml. (1000 International Units per ml.) of chorionic gonadotropin in isotonic saline daily for three days. The animals were sacrificed on the fourth day after receiving one of the radioactive preparations.

Radioactively labeled preparations (hormonal or control substances) were injected intracardially into the rats while they were under nembutal (4 to 9 milligrams per animal) anesthesia. Each animal received from 1 to 1000 micrograms of protein preparation.

** Bovine serum albumin (Lot J4902) of the Armour Laboratories was used throughout this study.

The animals were sacrificed by exsanguination from the dorsal aorta after various periods of time (3, 10, 30, 100 or 300 minutes). Perfusion of the animals was performed through the same needle that was used for exsanguination. Animals were perfused slowly with about 100 ml. of isotonic saline forced into the circulatory system from a hypodermic syringe.

A vaginal smear was made of each animal. Representative organs: (ovary, adrenal, blood, thyroid, kidney, liver, spleen, muscle, pancreas and mammary tissue) were removed, weighed, and placed either in Bouin's Solution or in 1 ml. of a 0.1N NaOH solution. The tissues that were placed in NaOH were digested for 16 hours at 100° C. These samples were transferred to steel planchets, dried and the radioactivity determined with an end window Geiger-Mueller tube. The radioactivity of the tissues in Bouin's Solution

TABLE 2. LOCALIZATION OF RADIOACTIVITY AT DIFFERENT TIMES AFTER THE ADMINISTRATION OF A LABELED PROLACTIN PREPARATION (PROLACTIN I^{131} No. 6)

Time in minutes	Ovary		Adrenal		Mammary gland*		Liver		Kidney	
	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood
3	1.48	0.33	1.50	0.33	0.18	0.05	1.48	0.33	9.31	2.1
10	2.54	1.03	1.45	0.59	0.19	0.08	3.43	1.40	29.25	11.9
30	2.27	1.55	1.13	0.77	0.24	0.09	2.95	2.01	20.25	13.8
100	1.10	1.25	0.40	0.46	0.11	0.11	1.08	1.22	2.75	3.1
300	0.40	1.22	0.20	0.60	0.07	0.11	0.43	1.30	2.25	3.8

Time in minutes	Spleen		Thyroid		Pancreas		Muscle		Blood
	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood	I^{131} conc.	Organ Blood	I^{131} conc.
3	0.68	0.15	0.89	0.19	0.63	0.14	0.08	0.02	4.54
10	1.38	0.56	1.75	0.72	0.42	0.29	0.11	0.05	2.46
30	1.59	1.08	24.20	16.40	0.87	0.59	0.20	0.14	1.47
100	0.67	0.77	153.50	180.0	0.36	0.41	0.13	0.15	0.88
300	0.26	0.79	312.50	1110.0	0.19	0.59	0.09	0.28	0.33

* Mammary gland studied in another experiment (Prolactin I^{131} No. 9), where the blood concentrations were similar to Prolactin I^{131} No. 6. No mammary tissue was removed from the animals receiving Prolactin I^{131} No. 6. The I^{131} concentration is expressed as the per cent of the administered dose which localizes per gram of tissue. The organ/blood ratio is the relationship between the concentration of I^{131} in the tissue to that in blood at the same time.

was assayed by gamma ray measurement in a multiple center wire counter (Marinelli and Hill, 1948). These tissues were then embedded in paraffin and cut in 10 micra sections for radioautography (Hamilton, Soley and Eichorn, 1940; Evans, 1947).

The results of the localization studies are expressed as the fraction of the administered dose which localizes per gram of tissue. In addition, the concentration of I^{131} in tissue is related to the concentration in blood and is indicated as the organ/blood ratio.

EXPERIMENTAL RESULTS

The biological activity of our labeled prolactin preparations, as measured by their ability to increase the crop sac weights of pigeons, has apparently been retained (Table 1). Whether these were labeled with an iodine to protein molecular ratio of 1 to 6 or 1.6 to 1, the preparations demonstrated as much biological activity as an unlabeled prolactin preparation. To determine the iodine to protein molecular ratios, the assumption was made that we had a homogeneous protein of average molecular weight of 30,000 (White, 1949).

Following the intracardiac administration of an I^{131} labeled prolactin

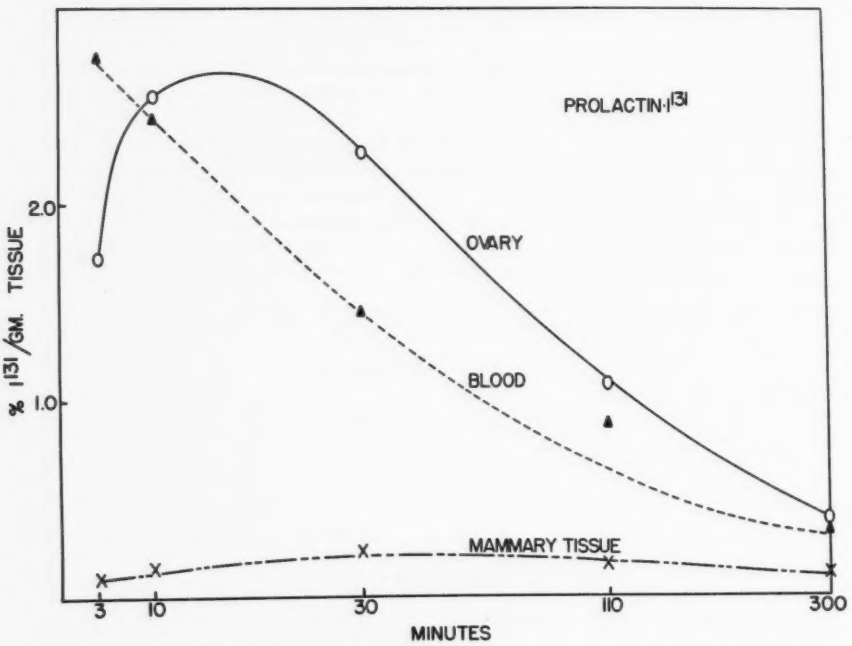

FIG. 1. Relationship of time and concentration of radioactivity in ovary, blood, and mammary tissue after the administration of labeled prolactin preparations. Semi-logarithmic scale.

preparation to the normal female rat, radioactivity was detectable in most of the removed tissues (Table 2). Soon after the injection of radioactive prolactin preparations, there was little concentration of radioactivity in the thyroid. However, this increased rapidly when longer periods elapsed between the time of injection and the time of sacrifice of the animals. In the ovary, the degree of localization of radioactivity increased rapidly and reached a maximum about twenty minutes after intracardiac injection. The concentration of radioactivity in the ovary at thirty minutes was significantly greater than blood (Fig. 1 and Table 2). The radioactivity decreased at a slower rate and there was still a significant concentration of radioactivity five hours after the administration of the radioactive prolactin preparations. As determined by vaginal smears, there was no correlation between the estrus cycle and the degree of localization of radioactivity in the ovary. There was no significant difference in the concentration of I^{131} in the ovary between animals pre-treated with chorionic gonadotropin and non-treated animals.

The administration of control substances, inorganic iodide labeled with

I^{131} and an I^{131} labeled bovine serum albumin (BSA) preparation demonstrated significantly less localization of radioactivity in the ovary at 30 minutes (Fig. 2 and Table 3). The pattern in the other organs was similar to that observed previously in male rats of the same strain (Sonenberg, Keston and Money, 1951).

Eighty-five per cent of the radioactivity in the ovary remained after perfusion when the animal received the radioactive prolactin preparation (Fig. 2 and Table 3). In five experiments with 24 animals, the amount lost by perfusion varied from 0 to 30%. In three of these experiments, the results were essentially identical with only 15% of the radioactivity in the ovary being lost by perfusion. After the administration of an I^{131} labeled bovine serum albumin preparation, only 10% of the radioactivity which localized in the ovary was retained after perfusion. This was essentially reproducible in 2 experiments with 10 animals. In the case of inorganic iodide labeled with I^{131}, approximately half of the radioactivity remained after perfusion. In 2 experiments with 16 animals, this finding was reproducible. In these perfusion studies, the animals were sacrificed 30 minutes after injection.

When the amount of protein injected was either 1, 10, 100 or 1000 micrograms, there was no significant difference in the degree of localization of radioactivity in the ovary at thirty minutes.

After the administration of radioactive prolactin preparations, the radio-

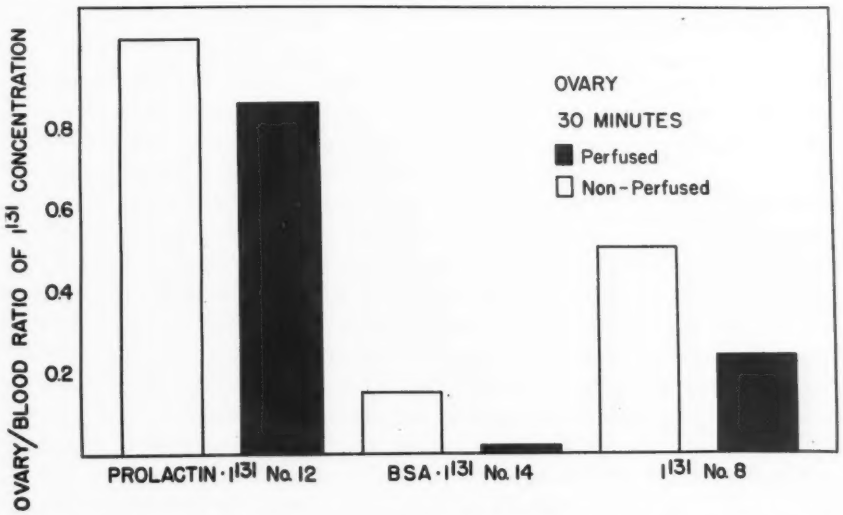

FIG. 2. Effect of perfusion on the localization of radioactivity in the ovary 30 minutes after the administration of I^{131} labeled substances.

TABLE 3. EFFECT OF PERFUSION ON THE LOCALIZATION OF RADIOACTIVITY IN THE OVARY 30 MINUTES AFTER THE ADMINISTRATION OF I^{131} LABELED SUBSTANCES. THE CONCENTRATION IS EXPRESSED AS THE % OF THE ADMINISTERED DOSE WHICH LOCALIZED PER GRAM OF OVARY

Preparation	Concentration of I^{131}		Ovary/blood ratio of I^{131} concentrations	
	Non-perfused	Perfused	Non-perfused	Perfused
Prolactin I^{131} #12	1.02	0.87	1.07	0.84
BSA I^{131} #14	0.35	0.04	0.15	0.02
I^{131} #8	0.51	0.29	0.51	0.24

activity in the mammary tissue did not suggest specific localization. The concentration of radioactivity demonstrated no significant differences from that found when I^{131} labeled iodide or a radioiodinated bovine serum albumin preparation was administered.

This lack of significant localization in the mammary tissue was obtained in normal adult and young female rats. Pregnancy or lactation in these rats did not alter the localization of radioactivity after the administration of a radioactive prolactin preparation.

In the radioautographic studies done with non-perfused animals, most organs gave a faint image when the tissue was apposed to a photographic emulsion (Hamilton, Soley, and Eichorn, 1940). However, when tissue sections were placed directly on the photographic emulsions (Evans, 1947), only the thyroid and kidney consistently showed heavy darkening of the photographic emulsion. Liver, spleen and pancreas showed considerable focal evidence of radioactivity with the latter method. Radioautographs of the ovaries were made, after the administration of six different labeled prolactin preparations. With four, localization was essentially vascular. With one preparation, radioactivity was localized to the luteal cells (Fig. 3), and in another, concentrated primarily in follicular fluid although also present in stromal vessels and in the central vessels of the corpora lutea. Concentration of the isotope in the thyroid was in the follicles and in the kidney was localized to convoluted tubules.

Radioiodinated bovine serum albumin studies showed radioactivity in many tissues when histologic sections were apposed to photographic emulsions. Direct contact of tissue and emulsion revealed heavy concentrations of radioactivity in the follicles of the thyroid and in the convoluted tubules of the kidney. There was localization of radioactivity to the ovary in one of three preparations studied. With this preparation, moderate concentration of the isotope was present in the follicular fluid, in stromal vessels, and central vessels of some corpora lutea. Concentration of I^{131} in the liver and spleen was focal and not consistently related to histologic structures.

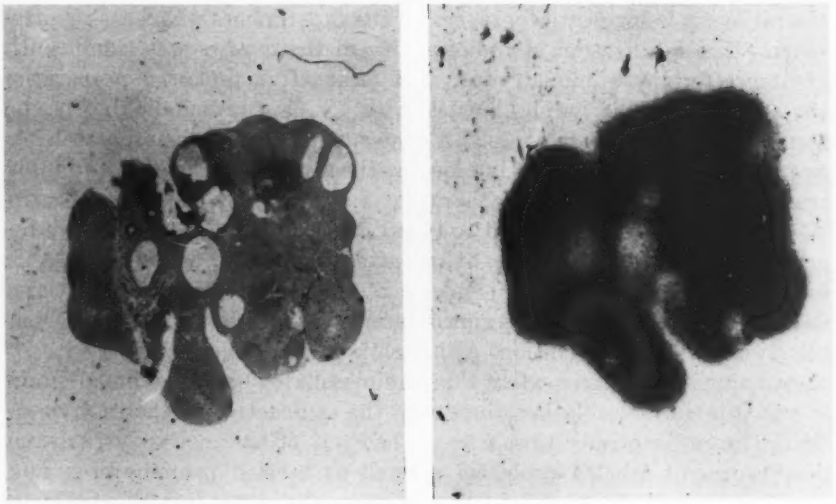

FIG. 3. Radioautograph of ovary removed thirty minutes after the administration of an I^{131} labeled prolactin preparation. Standard histological section on right with radioautograph on left. Dense areas correspond to corpora lutea and vacant areas to the follicles.

DISCUSSION

When only a small percentage of the total number of protein molecules has been reacted with the labeling reagent, one would not expect a loss of biological activity. Our radioiodinated prolactin preparations have retained their biological activity. This is significant in the case of our more heavily labeled preparation (1.6 to 1). An iodinated prolactin preparation with an iodine to protein molecular ratio of 1:1 or 2:1 has recently been shown (Cox, 1951) to be only slightly less biologically active than its non-iodinated control. The study of the biological activity of an iodinated protein is, however, limited by the degree of homogeneity of the protein preparation. The prolactin preparation used in this study was known to be contaminated with adrenocorticotropic activity. For such a non-homogeneous protein preparation, the rates of labeling each constituent may vary greatly (Pressman and Sternberger, 1950). Notwithstanding, it has been found (Li, Lyons, Simpson, and Evans, 1941) that it was necessary to introduce 12 molecules of iodine per protein molecule (calculated on the basis of 5% iodination and a homogeneous protein of 30,000 molecular weight) before there was reduction of the biological activity of prolactin. This strongly suggests that the molecules of our preparations were still biologically active.

The specificity of the bulk of the radioactivity which has localized in

the rat ovary is suggested by several of the experiments which are reported herein. The localization of radioactivity in the ovaries was significantly greater after the administration of a radioactive prolactin preparation than with inorganic iodide, labeled with I^{131} or a radioiodinated bovine serum albumin preparation. The concentration of radioactivity in the ovary was greater than that in blood at the same time. After the administration of control substances, there was a significantly smaller concentration in the ovary as compared to blood. The fact that 85% of the radioactivity was retained in the ovary after perfusion suggests a specific binding of the material. Non-specific radioactivity, like that associated with an I^{131} labeled bovine serum albumin preparation was almost completely removed by perfusion. Although inorganic iodide labeled with I^{131} was retained to a greater extent than radioiodinated bovine serum albumin, it was still considerably less than with the radioactive prolactin preparation. The radioactivity that was washed out of the ovary by perfusion may represent labeled prolactin as well as labeled proteins of a non-specific nature that were originally present in the inhomogeneous prolactin preparation. In addition, the radioactivity in the perfusate may represent the I^{131} from some degradation products of labeled prolactin as well as the products of I^{131} metabolism in the rat.

After the administration of one radioactive prolactin preparation, the ovaries showing localization of radioactivity revealed that the concentration was in the cells of corpora lutea. This is in accord with known effects of prolactin preparations. Prolactin has been shown (Evans *et al.*, 1938; Evans *et al.*, 1941 a, b) to maintain a secreting corpus luteum. The radioautographs with the I^{131} labeled bovine serum albumin preparation did not show the localization of radioactivity in the cells of the corpora lutea.

The half time of disappearance of radioactivity from the ovary of the rat after reaching a maximum is about ninety minutes. During the same period of time, the concentration of I^{131} in the blood fell with a similar rate. It is interesting to compare this with the much more rapid half time of disappearance of ACTH from plasma (Greenspan, Li, and Evans, 1950) of 5.5 minutes. After the administration of a radioactive ACTH preparation (Sonenberg, Keston, and Money, 1951), it was noted that the radioactivity in the adrenal diminished to half of the maximum value recorded in about 5.5 minutes.

The small concentration of radioactivity, both by direct measurement and by negative radioautography, in the mammary tissue of the rat after the administration of a radioactive prolactin preparation is somewhat surprising. In an inhomogeneous hormone preparation, the factor acting on mammary tissue may represent a small fraction of the preparation used.

This would then limit the degree of localization of the specific factor. The low concentration of I^{131} may be the result of including large amounts of fat and other non-mammary tissue with the mammary tissue. Since radioautographs were made using fixed tissues, there is the possibility of loss of radioactivity during the fixing procedures. In mice, after the administration of an I^{131} labeled prolactin preparation (Cox, 1951), "appreciable amounts of radioactivity were present in mammary gland and milk." Prolactin preparations will induce mammary growth in normal female rats as well as in ovariectomized rats (Riddle, 1947). When male rabbits are prepared with estrogen, prolactin preparations may induce alveolar hyperplasia (Lyons, 1936–1937). The local intraductal administration of prolactin preparations into hypertrophied mammary glands elicits lactation in the localized sector (Lyons, 1942). In pregnant and pseudopregnant rabbits, the intraductal injection of prolactin preparations into localized sections of the mammary gland induced lactation (Meites and Turner, 1947). This was noted to a lesser extent with whole pituitary extract. No effect was found with thyrotropin, thyroxine or adrenal cortical extract. From our experiments, we do not feel we have sufficient evidence to conclude that in the rat prolactin preparations act directly on mammary tissue or are fixed by it. The degree of maturity, pregnancy, or lactation did not seem to alter this finding.

A significant localization of radioactivity was found in the adrenal after the administration of a radioactive prolactin preparation. This is not surprising in view of the known contamination with adrenocorticotropic activity although the possibility that this adrenal localization is due to the prolactin alone has not been excluded. The disappearance rate of radioactivity from the adrenal is significantly different after the administration of a radioactive prolactin preparation than a labeled ACTH preparation. The presence of radioactivity in the liver and spleen may merely represent trapping by the reticulo-endothelial system. The localization of I^{131} in the liver may be specific inasmuch as prolactin preparations will increase the lipide concentration of the liver of pigeons (Riddle and Bates, 1939; Riddle, 1947). In pigeons, as well as mammals, this hormone is reputed to have an effect on the pancreas (Miller, 1942, Fraenkel-Conrat, *et al.*, 1942; Funk, *et al.*, 1941). We have noted no localization of radioactivity in the pancreas after the administration of a radioactive prolactin preparation which differed from the degree of localization with radioiodinated bovine serum albumin or I^{131} labeled inorganic iodide. It may well be that in some species, prolactin may have a primary effect on the viscera. However, in the evaluation of experiments using pituitary preparations, one must constantly be aware of the possibility that a contaminant in such a

preparation is inducing the observed effects. Actually, only a small portion of even a crystalline preparation may represent the biologically active material.

The high concentration of radioactivity in the kidney and its localization in the convoluted tubules by radioautography may represent a non-specific excretory process. We have noted a high degree of localization of radioactivity in the kidney, after the administration of all of our radioactive anterior pituitary preparations and our radioiodinated bovine serum albumin preparations. However, a specific effect of prolactin on the kidney has not been excluded.

In view of the relative absence of localization of radioactivity in the thyroid at the earlier times by direct radioactivity measurements and its rapid increase as the concentration in the other organs decreased, it is likely that this represents localization of nonprotein bound I^{131}. It would appear that as the I^{131} labeled prolactin preparation is degraded, some of the unbound I^{131} is taken up by the thyroid.

The same considerations (Sonenberg, Keston, and Money, 1951) may be invoked with our prolactin preparations as were employed to account for the small degree of localization of a biologically specific material like ACTH. Lack of homogeneity, rapid metabolism of I^{131} labeled prolactin by ovary and other tissues, and limited blood flow would restrict the degree of localization of radioactivity in the ovary. It is not likely that the disappearance curves can be related to the metabolism by one particular organ alone. As previously suggested (Sonenberg, Keston, and Money, 1951), the time curves may be a composite of labeled prolactin destruction by other tissues as well as ovary, the metabolism of unbound radioactive iodine and the metabolism of iodinated protein of a non-specific nature as well.

The results reported herein suggest that a labeled pituitary preparation may be used as a physiological tool. However, the limitations of the techniques, the inhomogeneity of the pituitary preparations, biological variations and other unknown variables demand caution in interpreting data obtained with such a tool. Further study is in progress in this laboratory in an attempt to increase the reliability of the method.

SUMMARY

An anterior pituitary preparation with prolactin hormonal activity has been labeled with I^{131}. The biological activity of our labeled preparations has been retained. The localization of radioactivity has been demonstrated in the ovary, adrenal, liver, spleen, and kidney after the administration of a radioactive prolactin preparation. There has been no significant concentration of radioactivity in mammary tissue of the rat under varying

physiological conditions. By radioautography, heavy radioactivity has been demonstrated in the thyroid and kidney and significant concentration in the corpora lutea of the ovary in one case and in the follicular fluid of the ovary of another.

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THE EFFECT OF THE INTRASPLENIC OVARIAN GRAFT ON PITUITARY GONADOTROPINS*

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INTRODUCTION

THE formation in the rat of a luteinized tumor from an ovary transplanted into the spleen at time of castration raises important questions concerning the pituitary-ovarian relationship.

Previous investigators have shown that the increase in circulating gonadotropin resulting from gonadectomy is due mainly, if not wholly, to an elevation of FSH (Witschi and Levine 1934, Biddulph *et al.* 1940, Biddulph and Meyer 1946). The LH component accumulates and is stored in the pituitary gland itself (Lipshutz and Villagran 1936, Hellbaum and Greep 1943, Greep and Hellbaum 1940). Different techniques have been used to demonstrate these clearly defined post-castration changes. Parabiosis experiments provide a measure of the circulating gonadotropins that have been, or are being discharged from the hypophysis whereas bioassay of the gland using immature recipients measures its gonadotropic content at time of autopsy.

The possible influence of an intrasplenic ovary on pituitary storage or release of gonadotropins after castration has been studied by both these methods. Thus, Miller and Pfeiffer (1950) demonstrated gonadal hypertrophy in an intact parabiont united to a grafted castrate litter mate, concluding that the presence of an intrasplenic ovary in a castrated mouse did not prevent an increased rate of production of circulating gonadotropins. Others (Jungck *et al.* 1947) have shown in the rat by bioassay of the pituitaries of single castrates bearing similar transplants that there is a marked reduction of gonadotropin potency compared with non grafted castrate controls. There is thus an apparent conflict in these two reports.

In order to characterize the simultaneous effects of an intrasplenic ovary on gonadotropin levels in the circulation and the pituitary gland,

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we have united 29 adult rats six weeks post castration in parabiosis for 12 to 26 days with intact partners. Eighteen of these castrates bore an intrasplenic graft implanted at time of ovariectomy. At autopsy, the pituitaries of all the castrates were assayed for gonadotropin content in immature recipients, while the weights of ovaries and uterus of the intact parabiont were recorded as a measure of increased gonadotropins in the circulation.

Since the pituitary hormone content of the castrate member of these pairs might be influenced by a cross-over of ovarian hormones from the intact partner, thirty-three single castrates were also prepared, thirteen of which bore an intrasplenic ovarian graft for from 8 to 48 weeks before autopsy. The potency of these animals' pituitaries were similarly assayed and served as controls to evaluate any possible "cross over" effect from the parabiotic union. The pituitary gonadotropin content of all castrates was measured against a standard derived from the response of immatures injected with the pituitaries of 17 normal adult rats.

The results confirm both the previous apparently contradictory reports and suggest a solution that may explain the occurrence of early and persistent luteinization in an ovary transplanted to the spleen of a castrate rat.

MATERIALS AND METHODS

Albino female rats originally from the Wistar strain, weighing 160–200 gms., were used for controls, for castration, and parabiosis. Immatures from the same colony[3] weighing 30–40 gms., were used for recipients in bioassay of the pituitaries.

Transplantation of a whole ovary to the spleen was done simultaneously with castration. In order to minimize the occurrence of lateral adhesions 0.6 mgs. of heparin were instilled into the abdominal cavity at closure following the suggestion of Van Dyke (1950) that this decreased the incidence of abdominal adhesions. In this group only 20% developed adhesions.

Twenty-nine parabiotic pairs were joined 6 weeks after preparing the castrate member with or without simultaneous ovarian transplant to the spleen. The technique of parabiosis was essentially that of Bunster and Meyer (1933). 10,000 units of crystalline penicillin was given each pair the day of operation and the first post-operative day. One pair died of pneumonia on the 12th post-operative day in spite of aureomycin administered by gastric tube. Two others succumbed at 14 days while the rest were forfeited 16 to 26 days after operation. The animals were killed with illuminating gas, separated, and individual body weights recorded. The ovaries of the intact parabiont were dissected free and weighed. The uteri of both rats were opened and blotted to remove free fluid, then weighed on a torsion balance.

The pituitary of the castrate parabiont was then dissected with care and immediately homogenized in 6 cc. of sterile physiologic saline. Bidaily subcutaneous injections of 1 cc. for 3 days were started the same day in a weanling recipient which was sacrificed on the 4th day, 24 hours after the last injection. Each assay rat thus received the whole of an

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TABLE 1. EFFECT OF AN INTRASPLENIC OVARIAN GRAFT ON LEVEL OF CIRCULATING GONADOTROPINS IN A CASTRATE MEASURED BY INCREASE OVER NORMAL OF OVARIAN WEIGHTS OF AN INTACT PARABIONT AFTER 2 TO 3 WEEKS

	No.	Average body wt. (gms.)	Mean ovarian wt. ± S.E. (mgm.)	Ratio to control	51 to 60	61 to 70	71 to 80	81 to 90	91 to 100	101 to 110	111 to 120	121 to 130	131 to 140	141 to 150	Weight over 150
Intact adult controls	17	168	76.2± 4.9	—	1	5	5	3	3						
Intact parabiont united to castrate	11	147	117.0± 21.9	154%	1	3			1	1		1	2	1	282
Intact parabiont united to castrate with ovary in spleen	18	179	122.0± 17.6	160%	2		2	4	1	1	1	2			172 175 192 205 317

adult pituitary. The weights of ovaries and uterus of the recipient were recorded. Hyperemia of the ovaries was noted as 0 to ++++. Representative specimens of ovaries or uterus were fixed, sectioned and stained for microscopic study with hematoxylin and eosine.

RESULTS

In Table 1 it is seen that the average ovarian weight of the intact parabionts united to castrates for 12 to 26 days was approximately 50% or more greater than that of intact controls whether or not the castrate partner contained a growing, luteinized ovarian transplant in the spleen. This indicates that no decrease in circulating gonadotropins is associated with a transplanted ovary in the spleen of a castrate.

Quite different effects of such an ovarian graft are noted in Table 2 which records gonadotropic pituitary content. Recipient ovarian weights

TABLE 2. EFFECT OF AN INTRASPLENIC OVARIAN GRAFT ON GONADOTROPIN CONTENT OF CASTRATE PITUITARY MEASURED BY INCREASE OF RECIPIENT OVARIAN WEIGHTS OVER THAT INDUCED BY ASSAY OF NORMAL ADULT PITUITARY

Non-grafted controls	No.	Average body wt. of recipients (mgs.)	Mean ovarian wt. (mgs.) of recipient, ± S.E.	Ratio to control	11 to 20	21 to 30	31 to 40	41 to 50	51 to 60	61 to 70	71 to 80	81 to 90	91 to 100	101	Remarks
Recipient of intact adult controls	17	43.8	21.2± 1.6	—	8	9									
Recipient of castrate controls	20	44.0	85.0± 5.1	401%					1	2	7	2	6	3	
Recipient of castrates in parabiosis	11	43.6	75.0± 10.7	354%				2	3	1		1		4	Ovaries show +++ to ++++ hyperemia, early luteinization
Totals for all ungrafted castrates	31	44.1	81.3± 7.2	383%				2	4	3	7	3	6	6	
Splenic ovarian grafts															
Recipient of castrate with ovary in spleen	13	44.6	44.0± 9.2	208%	3	4	1		1	1		2	1		
Recipient of castrate parabiont with ovary in spleen	16*	48.0	47.0± 6.6	222%		6	1	4	1		2	2			Ovaries show 0 to ++ hyperemia, well-developed luteinization
Totals for all castrates with ovary in spleen	29	48.0	45.9± 5.1	217%	3	10	2	4	2	1	2	4	1		

* The pituitaries of only 16 of the 18 castrate parabionts with ovarian grafts were assayed.

TABLE 3. SUMMARY OF EFFECT OF INTRASPLENIC OVARIAN TRANSPLANT ON CIRCULATING GONADOTROPINS AND PITUITARY POTENCY MEASURED SIMULTANEOUSLY IN 29 PARABIOTIC PAIRS OF RATS BY OVARIAN WEIGHTS ALONE

	Castrate controls (11 pairs)	Castrate with intrasplenic ovary (18 pairs)
Increase in circulating gonadotropin (ratio to intact parabiont ovarian weight)	154%	160%
Increase in pituitary potency (ratio to control recipient ovarian weight)	354%	222%*

* Only 16 out of 18 animals assayed.

obtained by assay of the pituitaries of 31 castrates without intrasplenic grafts gave an average of 383% increase over the weights obtained by similar injections of 17 normal control pituitaries. The 11 parabionts among these 31 ungrafted castrates showed only a little less increase (354%) than that found in the 20 castrate controls (401%). This indicates very little if any "cross over" inhibition, by the intact rat's ovarian secretions, of the castrate partner's pituitary in 2 to 3 weeks. However, in the presence of an intrasplenic ovary, the assay of the castrate pituitary showed significantly reduced gonadotropic potency. These 29 animals

TABLE 4. UTERINE WEIGHTS OF CASTRATES, OF INTACT PARABIONTS AND OF IMMATURE RECIPIENTS OF CASTRATE PITUITARY ASSAYS

	No.	Average body weight, gm.	Average uterine weight, mg.	Ratio to intact adult controls
Intact adult controls	17	168	273	—
Castrates:				
Castrate controls	20	247	48.6	18%
Castrate with ovary in spleen	13	276	85	31%
Castrate parabiont	11	181	75.5	27%
Castrate parabiont with ovary in spleen	18	188	103.0	38%
Intact parabionts:				
Intact adult controls	17	168	273	—
Intact parabiont of simple castrate	11	147	356	130%
Intact parabiont of castrate with ovary in spleen	18	179	437	160%
Immature recipients:				
From normal adult controls	17	43.8	61.2	—
From castrate controls	20	44.0	90.0	147%
From castrate with ovary in spleen	13	44.6	82.2	134%
From castrate parabiont	11	43.6	86.0	141%
From castrate parabiont with ovary in spleen	16	48.0	106.0	173%

caused an ovarian recipient increase of only 217% over normal controls. No important difference is noted when the average of the 13 single grafted castrates (208% of normal) is compared with that of the 16 grafted castrate parabionts (222% of normal). It can therefore be concluded that any cross-over effect from the intact parabiont on the potency of the partner's pituitary was negligible.

Table 3 summarizes the significant results from the two previous tables in our parabiotic pairs on the basis of ovarian weights alone. It seems clear that an intrasplenic graft does not decrease the level of circulating gonadotropins but at the same time it does reduce the gonad stimulating ability of the pituitary gland itself.

The uterine wet weights of all the animals are recorded in Table 4.

The uteri of the intact parabionts united to simple castrates showed varying degrees of distention with fluid. When this was released, they

FIG. 1. Uterus of immature recipient injected with pituitary of splenic transplant parabiont. Little or no intraluminal fluid was present. The lumen is small, and there is a thick sub-endometrial layer with dense stroma, causing opacity. This is consistent with progesterone stimulation.

FIG. 2. Uterus of immature recipient injected with pituitary of simple castrate parabiont. Following release of fluid, there is festooning of the luminal margin with an increased surface area. The sub-endometrial layer is thin and edematous so that the endometrium lies close to the muscularis and gives rise to translucency in the gross. This may be interpreted as the effect of estrogen only.

weighed 30% more than the average for the controls. When an ovary was grafted into the spleen of the castrate, then the uteri of the intact parabionts were in general opaque, contained little fluid, and weighed 60% more than the controls.

There is also seen in Table 4 an increase of uterine weight in the immature recipients of all the castrate pituitaries assayed, when compared with the weight induced by injection of the normal adult pituitary. This is to be expected as a reflection of the action of elevated gonadotropins in the castrate on estrogen production in the immature ovary. The uterine weights of the recipients from castrate parabionts with grafts show a greater increase than do those of the other groups. Microscopic sections of the recipient's ovaries in this group showed many well developed corpora lutea. The uteri of these same immature animals were generally thick-

walled with a dense stroma and an endometrium suggesting diestrus[4] (Fig. 1).

Grossly, they were opaque, and had little or no intraluminal fluid. This appearance has been considered (Maddock *et al.* 1949) to be typical of the effect of progesterone added to estrogen, and has been used to demonstrate the action of luteotropin as well as FSH and LH on the animals' ovaries. In contrast, the uteri of the immature assay animals for the pituitaries of the simple castrate parabionts were thin-walled with large lumina and an endometrium of the late estrus type (Fig. 2). They appeared translucent, distended with fluid. Such findings have been associated with estrogen elaboration only from the ovary indicating the stimulation of FSH and LH but not of luteotropin (Maddock *et al.* 1949).

It is interesting to observe (see Table 2) that the ovaries of the immature recipient of the simple castrate parabiont's pituitary showed generally a well marked hyperemia, but only very early luteinization, while those in the recipient of the grafted castrate parabiont's pituitary showed very little or no hyperemia but mature corpora lutea.

DISCUSSION

It is clear that in such a dynamic system as the pituitary-ovarian relationship, the time factor must be carefully considered in interpretation of observed changes. Especially is this true, as shown in the basic observations of Biskind & Biskind (1949) of changes during the first few days after castration and ovarian transplantation to the spleen.

These workers have shown that new primordial follicles appear in 3 or 4 days, with luteinization occurring by the fifth or sixth day. These corpora lutea as well as those that continue to develop do not involute but remain morphologically unchanged for 150 days.

All of our parabiotic pairs were united six weeks after castration and ovarian transplantation, and only those with well advanced and healthy looking ovarian tumors were selected for parabiosis. Among our group of single castrate controls with intrasplenic ovaries, the duration of the graft varied from 56 to 336 days. When the pituitaries of this group were assayed for gonadotropic potency, however, we could not correlate any significant finding with duration of the transplant within these time limits.

Several investigators have shown in the rat that 10 days of parabiotic union is sufficient for an intermingling of the circulation that produces demonstrable cross-effects in the opposite partner (Meyer and Hertz

[4] It is difficult to form conclusions concerning estrogenic or progestational effects from the histology of the endometrium in the rat. However, through the courtesy of Dr. F. L. Hisaw we have been privileged to compare our sections with the exhaustive but unpublished studies of D. D. Kozoll (1936) upon which the above statements are founded.

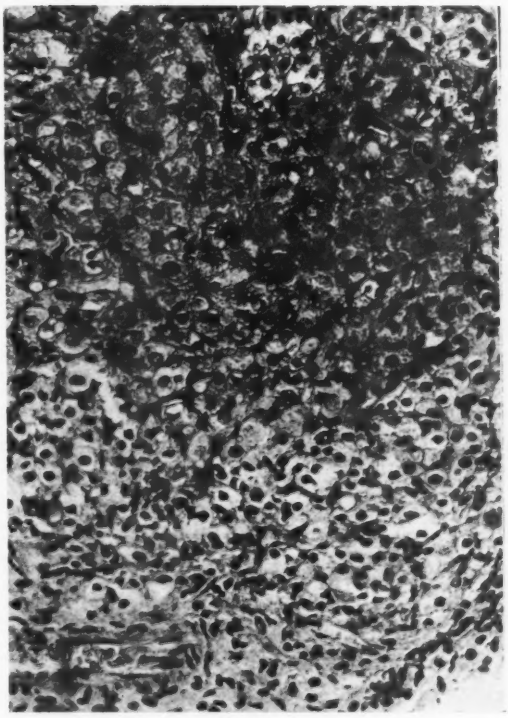

FIG. 3. High power view of luteoma in spleen showing cells similar to those of a normal functioning corpus luteum.

1937, Biddulph *et al.* 1940, Biddulph and Meyer 1946, Byrnes *et al.* 1951). In this interval the union of a castrate to a hypophysectomized rat produced follicles but no corpora lutea proving that FSH and no LH was discharged into the circulation (Biddulph and Meyer 1946). We have allowed an average of approximately 21 days in parabiosis. Storage of LH in the pituitary of the rat has been demonstrated 15 days after castration (Hellbaum and Greep 1943, Byrnes *et al.* 1951). If after an animal is castrated estrogen is given, bioassay of the pituitary no longer shows storage of LH. A number of workers agree that this finding is due to the fact that estrogens cause a discharge of LH from the hypophysis of the laboratory animal (Greep and Hellbaum 1940, Biddulph and Meyer 1946, Greep and Hellbaum 1946, Smith 1945, Hellbaum and Greep 1943, Wolfe and Brown 1942, Biddulph *et al.* 1940, Fevold, Hisaw and Greep 1936). There is also evidence that a release of LH from the pituitary of postmenopausal women can be produced by estrogens and that this can be detected

in the urine of these patients by biologic methods (Lloyd *et al.* 1944, Funnel *et al.* 1951). Smith (1945) reported that Westerfeld's Lactone, a synthetic oxidation product of crystalline estrone, was even more effective in this regard than estrone, and advanced the hypothesis that some product of estrogen inactivation in the liver might be responsible for pituitary release of LH rather than the estrogen itself (Smith 1944).

A newly grafted intrasplenic ovary in a castrate responding to rapidly increasing FSH levels, produces a crop of new follicles which can be shown to be functional since the development of vascularized adhesions to the lateral abdominal wall allows ovarian secretions to enter the peripheral circulation and, under these circumstances, the pelvic organs do not atrophy but are normally maintained. When no such adhesions develop, all ovarian secretions from such transplants pour directly into the portal circulation. In such animals showing uterine atrophy only the non-estrogenic products of estrogen degradation are available to exert an effect on the pituitary.

Massive early luteinization of the intrasplenic graft appears to present impelling evidence that products of ovarian steroid degradation released by the liver cause a discharge of pituitary LH.

Luteinizing hormone alone, or in combination with FSH would not, however, allow the persistence of corpora lutea. Greep (1938) showed in the rat that corpora induced before hypophysectomy and usually persistent, could be wiped out by ten days' treatment with LH. Their complete involution was even more rapid when FSH was combined with LH. The progressive development of new crops of corpora for many weeks in the intrasplenic ovary and their apparent functional persistence strongly suggests that not only LH must be continuously available in the general circulation, but luteotropin[5] as well. This is consistent with Astwood's report (1941) that corpora induced before hypophysectomy in the rat would not be destroyed by daily injections of LH if luteotropin was given simultaneously.

The histologic appearance of luteinization in the transplanted ovaries is indistinguishable from that of normal functioning corpus luteum cells (Fig. 3). As in the normal cycle, one might expect that progesterone as well as estrogen may therefore be produced. Little is known of the metabolic pathway of progesterone although it has been assumed that this hormone, like estrogen, is probably broken down by the liver. The recent isolation of pregnanediol from human bile (Rogers and McLellan 1951) lends support to this view. In our experiments it is impossible to determine

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[5] Luteotropin is here used for convenience, rather than prolactin, with the recognition that most investigators consider the two terms interchangeable.

whether the effects that we interpret to be due to steroid breakdown products result from the metabolism of estrogen or progesterone or a combination of the two.

Luteomata do not develop in an intrasplenic ovarian graft if the other ovary is retained or estrogen given subcutaneously. Steroid hormones from the latter sources first are absorbed into the general circulation and can therefore produce an effect directly upon the hypophysis before their eventual inactivation in the liver. In the case of a splenic ovary in a castrate, however, all the steroid production concentrates directly in the liver, and uterine atrophy attests to its total inactivation there. It seems plausible to conclude that the abnormal concentration of break-down products of ovarian steroids from intrasplenic grafts may be responsible for the demonstrated changes in pituitary gonadotropins reported in our experiments.

It is interesting to note that a relative increase in uterine weight occurred in all castrate parabionts when compared with the weight of single castrate controls. This could be explained as an estrogen effect brought about by the intermingling of the circulation of the intact animal with that of its castrate partner. There is evidence to suggest that increase in uterine weight is a less sensitive measure of circulating estrogen than pituitary inhibition (Byrnes and Meyer, 1951). We might on this basis expect to find a reduction of pituitary potency in our parabiotic castrates due to estrogen cross-over, since their stimulated uteri already showed that some estrogenic response had been produced. A minimal reduction in potency did appear to occur in the animals without an intrasplenic ovary. However, no pituitary inhibition is detectable in the grafted castrate parabionts. The fact that the pituitary of a single castrate with intrasplenic ovary showed approximately the same reduction in potency as that of a grafted castrate parabiont appears to rule out any possibility that pituitary inhibition by estrogen crossing over the parabiotic union could have played an important role in interpreting our results.

Ovarian hyperemia was described by Zondek and Ascheim (1927) as the earliest of the reactions to the hormone of pregnancy urine. Recent investigation has demonstrated the great sensitivity of this reaction to hormone principles containing LH (Politou and Sturgis, 1951). Since LH accumulates in the castrate pituitary, the marked hyperemia in recipient ovaries noted in Table 2 is to be expected in comparison with a lesser degree seen to result from the castrate pituitaries whose stored LH has been depleted by an ovarian transplant. The explanation for more fully developed corpora lutea in the recipients of the grafted animals, however, is not clear.

Biskind observed (1949) that "the major factor concerned in the etiology

of this (intrasplenic) experimental tumor in rats is inactivation by the liver of hormones produced by the transplanted ovary." The answer to some of the problems raised by the formation of intrasplenic luteomata appears to lie in the effect on the castrate pituitary of certain of these ovarian inactivation products.

SUMMARY

The occurrence of early luteinization in an intrasplenic ovary in a castrated rat affords an opportunity to reappraise the mechanisms governing storage and release of pituitary gonadotropins and the interactions of ovarian and pituitary hormone secretions. The presence of such an intrasplenic ovarian transplant was found to reduce the ability of the pituitary to cause a recipient's gonads to hypertrophy. In the same animal, however, the level of circulating gonadotropins was found to have been maintained. These findings are considered consistent with the hypothesis that a functioning ovarian graft in the spleen is responsible for a release of gonadotropin—presumably LH—into the general circulation, whereas an accumulation or "storage" of this hormone occurs in the pituitary of the ungrafted castrate control. Since the transplanted ovary invariably showed persistent and progressive luteinization and eventual luteoma formation, it is apparent that luteotropin as well as LH must be continuously available from the pituitary of the grafted animals. Continued production of estrogenic steroids by the transplants is demonstrated in those that develop vascular adhesions to the abdominal wall. When such adhesions do not occur, the uterine atrophy in the grafted animals is considered evidence that all estrogenic compounds from the intrasplenic ovary were inactivated in the liver, and none escaped into the general circulation. It is suggested that the factors responsible for the release of LH and luteotropin under these circumstances must have been the degradation products of ovarian steroids produced by their break-down in the liver.

These findings are consistent with the suggestion of O. W. Smith (1944) that certain of the effects of estrogen on the pituitary of an intact animal may be accountable to the oxidative break-down products produced in the liver, rather than to estrogen per se.

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INHIBITION OF PROGESTERONE IN DECIDUAL DEVELOPMENT BY STEROID COMPOUNDS[1,2]

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THE inhibition of progesterone by estrogens has been reported by many investigators and the literature dealing with this reaction has been reviewed by Courrier (1950). More recently, we (Velardo and Hisaw, 1951) have made a comparative study of the inhibitory action of several estrogens on the decidual response in rats, and also have extended our observations to include other steroid compounds. We were interested particularly in natural and synthetic compounds that are more or less chemically related to progesterone and which are non-progestational in their action or are effective only at high doses. It was thought that certain of the inactive steroids might compete with progesterone and thus modify the development of deciduomata. The present paper reports the results of a series of experiments designed to test this possibility.

PROCEDURE

A standard procedure was established by first measuring the rate of decidual growth in the normal pseudopregnant rat, and then determining the daily dose of progesterone required for duplication of the normal response in the castrated animal. Pseudopregnancy was induced in estrous rats by electrical stimulation of the uterine cervix. The right horn of the uterus was traumatized on the fifth day of pseudopregnancy by passing a needle through the uterine lumen from tubal junction to cervix and withdrawing it slantwise, thus scratching the endometrium on the antimesometrial side throughout its entire length. The animals were killed 24, 48, and 72 hours after traumatization of the uterus and the diameter of both horns was measured *in situ*. It was found that with some practice deciduomata of quite constant diameters could be produced, thus making it possible to standardize the reaction in terms of a normal response. Deciduomata 24 hours after traumatization were 2.9 ± 0.20 in diameter and the contralateral horn 1.9 ± 0.17 mm. A reaction of this proportion was designated as +2. Uteri at 48 hours measured 3.6 ± 0.34 and 2.1 ± 0.19 mm., a +3 reaction, and those at 72 hours were 4.8 ± 0.35 and 2.2 ± 0.22 mm., a +4 reaction. Also, in experiments on inhibition, ques-

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tionable responses in which only a few decidual cells were present were designated as +1, and negative responses recorded as 0.

It was found that following castration and traumatization of the uterus on the fifth day of pseudopregnancy, a daily injection of 1.5 mg. of progesterone for three days would produce deciduomata approximately equivalent to those present at 72 hours in the intact animal. Such deciduomata had an average diameter of 4.5±0.25 mm., and the average for the untraumatized horn was 2.2±0.21 mm. Therefore, the routine followed in experiments on inhibition was to castrate the animal on the fifth day of pseudopregnancy, traumatize the right uterine horn, give three daily injections of 1.5 mg. progesterone plus the compound being tested and examine the uterus at the end of 72 hours. The steroids were dissolved in sesame oil and given in 0.1 ml. doses when possible. depending upon solubility of the compound.

RESULTS

Pregnanediol. This steroid is generally considered as being physiologically inert as it does not produce estrogenic or progestational effects nor can it substitute for adrenal cortical hormones (Selye, 1942). However, it is an effective antagonist of progesterone in the decidual reaction in rats. The effect of three daily injections of 1.5 mg. of progesterone which produces a +4 decidual reaction in the castrated pseudopregnant rat is completely inhibited when 0.25 mg. pregnanediol is given concurrently. It also seems that the inhibitory effect of pregnanediol is proportional to the dose as 0.1 mg. permits a +2 reaction, and 0.2 mg. a +1. A point of equal interest is that 12 mg. of pregnanediol daily for the three-day period consistently failed to induce decidual growth.

That the interaction between progesterone and pregnanediol is one of competitive inhibition is indicated by the fact that the reaction can be reversed. When the daily dose of pregnanediol (0.25 mg.) that is required

TABLE 1. INFLUENCE OF PREGNANEDIOL ON THE ACTION ON PROGESTERONE IN DECIDUAL DEVELOPMENT

Pregnanediol, daily dose in mg.	Progesterone, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
0.25*	1.5	0	1.8±0.13†	1.7±0.10
0.20	1.5	+1	2.6±0.20	1.9±0.14
0.10	1.5	+2	2.8±0.18	2.1±0.17
12.00	0.0	0	1.6±0.13	1.5±0.12
0.00	1.5	+4	4.5±0.25	2.2±0.21
		Reversal		
0.25	2.0	0	1.9±0.19	1.8±0.07
0.25	2.5	+1	2.5±0.10	2.2±0.11
0.25	3.0	+2	2.8±0.17	2.1±0.16
0.25	3.5	+3	3.8±0.32	2.2±0.16
0.25	4.0	+4	4.4±0.21	2.2±0.12

* Three to five rats per experimental group.
† Standard deviation.

to inhibit 1.5 mg. progesterone is kept constant and the dosage of progesterone increased, decidual development is progressively restored until +4 deciduomata are attained (Table 1).

Pregneninolone (*Ethinyl-testosterone*). This compound has weak estrogenic, androgenic, and progestational properties. Salmon and Salmon (1940) report that 0.5 mg. per day for three days will produce an estrogenic effect in the uterus and vagina of rats, and at the same time will show androgenic properties by promoting growth of the preputial glands and clitoris. In women, 60 mg. daily, when given orally, induces growth of the vagina but not of the uterus, but will promote a progestational reaction

TABLE 2. ANTAGONISM BETWEEN PREGNENINOLONE AND PROGESTERONE IN DEVELOPMENT OF DECIDUOMATA

Pregneninolone, daily dose in mg.	Progesterone, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
0.75*	1.5	+2	2.9±0.20	2.0±0.17
1.50	1.5	+2	2.8±0.15	2.0±0.13
3.00	1.5	+2	2.8±0.19	2.1±0.18
6.00	1.5	+1	2.5±0.16	2.1±0.17
12.00	1.5	+1	2.5±0.11	1.9±0.17
12.00	0.0	+1	2.4±0.15	2.1±0.19

* Three to five rats per experimental group.

in the endometrium when preceded by an estrogen treatment (Salmon and Geist, 1940). Courrier and Jost (1939) report that pregneninolone in 50 mg. doses daily will maintain pregnancy in spayed rabbits and is androgenic in castrated rats and in chicks.

In our experiments on the inhibition of decidual development it was found that pregneninolone had a definite antagonistic action on progesterone (Table 2). A daily dose of 3 mg. of pregneninolone reduced the effects of our standard dose of progesterone 50%, while 6 mg. decreased the reaction to +1. Further increase in the dosage of pregneninolone did not result in complete inhibition. This was probably due to the progestational action of pregneninolone itself as 12 mg. daily when given alone produced +1 deciduomata.

Pregnenolone. When tested on laboratory animals, pregnenolone produces several effects that are characteristic of estrogens, androgens, progesterone and adrenal cortical steroids (Henderson, Weinberg, and Wright, 1950). However, large doses are usually required for such effects. Selye and Masson (1943) found, in the McPhail rabbit test, that 40 to 100 mg. of pregnenolone was required to give a progestational reaction equivalent to that produced by 1.0 I.U. of progesterone.

Pregnenolone did not antagonize progesterone even when given in relatively large doses of 12 mg. daily and produced +2 deciduomata when given alone at this dosage (Table 3).

TABLE 3. PREGNENOLONE AND PROGESTERONE IN DECIDUAL DEVELOPMENT

Pregnenolone, daily dose in mg.	Progesterone, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
1.5	1.5	+4	4.7 ±0.27	2.2 ±0.20
3.0	1.5	+4	4.7 ±0.31	2.1 ±0.18
6.0	1.5	+4	4.5 ±0.25	2.2 ±0.12
12.0	1.5	+4	4.8 ±0.20	2.0 ±0.15
12.0	0.0	+2	2.7	2.2

Pregnanedione. This compound apparently is entirely devoid of estrogenic, androgenic, and progestational activity. However, Selye (1942) reports that of 75 steroids tested, it has the highest anesthetic power. When used as a competitor of progesterone it showed no inhibitory effect on decidual development even in doses of 12 mg. per day nor did this dosage when given alone produce growth of the uterus and vagina (Table 4).

TABLE 4. PREGNANEDIONE AND PROGESTERONE IN DECIDUAL DEVELOPMENT

Pregnanedione, daily dose in mg.	Progesterone, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
1.5*	1.5	+4	4.8 ±0.29	2.1 ±0.17
3.0	1.5	+4	4.6 ±0.16	2.0 ±0.13
6.0	1.5	+4	4.4 ±0.18	2.2 ±0.11
9.0	1.5	+4	4.8 ±0.21	2.2 ±0.09
12.0	1.5	+4	4.7 ±0.24	2.2 ±0.11
12.0	0.0	0	2.3 ±0.07	2.0 ±0.10

* Five rats per experimental group.

Testosterone. Several investigators have reported that large doses of testosterone produce weak to moderate progestational responses in experimental animals. Kline and Parkes (1937) found by using the McPhail method that 4 mg. daily for five days would produce a +1 or +2 progestational reaction in the immature rabbit. A daily dose of 9 mg. of testosterone propionate for five days will precipitate menstruation in castrated monkeys receiving 1,000 I.U. of estrogen daily. However, menstruation occurred in only one of two animals given 30 mg. testosterone daily for the same period. This is an effect that ordinarily requires only a single injection of 1 mg. of progesterone (Hisaw, 1943).

In our experiments on the inhibition of progesterone it was found that 1.0 mg. of testosterone daily would reduce the decidual response induced by 1.5 mg. progesterone 25%, and complete inhibition was attained by a daily dose of 1.5 mg. (Table 5). Testosterone at a daily dose of 12.0 mg. did not produce a decidual reaction and the traumatized and contralateral horns of the uteri had the same average diameters. It is a possibility

TABLE 5. PROGESTERONE INDUCED DECIDUOMATA INHIBITED BY TESTOSTERONE

Testosterone, daily dose in mg.	Progesterone, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
0.25*	1.5	+4	4.4±0.11	2.3±0.08
0.50	1.5	+4	4.6±0.22	2.1±0.18
0.80	1.5	+4	4.4±0.19	2.2±0.10
0.90	1.5	+4	4.5±0.17	2.0±0.10
1.00	1.5	+3	3.8±0.20	2.1±0.16
1.10	1.5	+3	3.9±0.15	2.1±0.18
1.25	1.5	+2	2.9±0.23	2.0±0.20
1.30	1.5	+1	2.5±0.19	1.9±0.17
1.40	1.5	+1	2.6±0.14	2.0±0.11
1.50	1.5	0	2.0±0.09	1.8±0.11
12.00	0.0	0	1.9±0.13	1.9±0.16

* Four to five rats per experimental group.

that the estrogenic action of testosterone might have contributed to the inhibition of progesterone. Nathanson *et al.* (1938) found that 2.5 to 10 mg. of testosterone propionate given as a single injection produced uterine growth and vaginal cornification in castrated immature rats. In a study of the antagonism of progesterone by estrogen in development of deciduomata in castrated pseudopregnant rats Velardo and Hisaw (1951) showed that 0.110 μg. estradiol 17-beta daily would completely inhibit a daily dose of 1.5 mg. progesterone. The amount of testosterone required for an estrogenic effect equivalent to that produced by 0.110 μg. estradiol has not been determined.

11-Desoxycorticosterone acetate (*DCA*). Progestational action of DCA has been reported by many investigators, most of whom found it 10 to 20 or more times less effective than progesterone (Selye, 1942). Masson (1943) found that rats castrated in estrus and given 10 mg. DCA daily for ten days showed a normal decidual response when the uterus was traumatized on the fifth day of treatment. When DCA was used as a competitor in decidual development only 3 mg. was required to completely inhibit the standard dose of 1.5 mg. of progesterone, and this amount when given alone was incapable of eliciting a decidual response (Table 6). In other experiments it was found that in sexually immature hypophysectomized rats 1.5 mg. DCA daily for four days increased the weight of the uterus

about 20%, 5 mg. daily almost doubled uterine weight, and 10 mg. daily produced only 25% increase. This observation and the fact that 1.5 and 3.0 mg. DCA daily for three days did not increase the diameter of the uterus (Table 6) indicate that the ability of DCA to inhibit progesterone in decidual development is not correlated with its capacity to promote uterine growth.

Cortisone (*Cortone acetate*). Experimental evidence at hand indicates that cortisone has very little if any direct effect on the male or female reproductive organs (Ingle, 1950). We have found that cortone acetate in doses of 1.5, 5.0, and 10 mg. daily for four days does not stimulate growth

TABLE 6. 11-DESOXYCORTICOSTERONE ACETATE AS AN INHIBITOR OF PROGESTERONE IN THE DEVELOPMENT OF DECIDUOMATA

Progesterone, daily dose in mg.	D.C.A., daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
1.5	0	+4	4.5±0.25	2.2±0.21
1.5*	0.5	+4	4.3±0.19	2.2±0.26
1.5	1.0	+3	3.6±0.24	2.1±0.15
1.5	1.5	+2	2.9±0.21	2.0±0.19
1.5	2.0	+2	2.8±0.22	2.1±0.16
1.5	2.5	+1	2.3±0.11	1.8±0.17
1.5	3.0	0	2.2±0.09	1.9±0.18
0	1.5	0	2.3±0.13	1.7±0.21
0	3.0	0	2.2±0.20	1.8±0.19

* Three to five animals per experimental group.

of the ovaries, uterus, or vagina in hypophysectomized sexually immature rats. Yet, a daily injection of 1.5 mg. of this compound will reduce the diameter of deciduomata induced by 1.5 mg. of progesterone in castrated pseudopregnant rats 25%, and 4.5 mg. daily will completely inhibit decidual development (Table 7).

TABLE 7. CORTISONE AS AN INHIBITOR OF PROGESTERONE IN DECIDUAL DEVELOPMENT

Progesterone, daily dose in mg.	Cortisone, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horn in mm.
1.5*	1.5	+3	3.7±0.23	2.2±0.16
1.5	2.0	+2	2.8±0.18	2.1±0.14
1.5	2.5	+2	2.9±0.11	2.1±0.14
1.5	3.0	+2	2.7±0.19	1.9±0.16
1.5	3.5	+1	2.5±0.07	1.7±0.13
1.5	4.0	+1	2.5±0.10	1.8±0.15
1.5	4.5	0	1.8±0.17	1.6±0.11
0.0	1.5	0	1.7±0.23	1.8±0.08

* Three to five animals per experimental group.

Adrenocorticotropic Hormone (ACTH). The inhibitory action of adrenal cortical steroids suggested the possibility that adrenal hormones secreted in response to ACTH may produce a similar effect. Therefore, castrated pseudopregnant rats were given graded doses of ACTH during the three days following uterine traumatization in conjunction with the standard daily dose of 1.5 mg. progesterone (Table 8). The decidual response was

TABLE 8. PROGESTERONE INDUCED DECIDUAL DEVELOPMENT INHIBITED BY ACTH

Progesterone, daily dose in mg.	ACTH, daily dose in mg.	Decidual rating	Mean diameters	
			Decidual horn in mm.	Contralateral horns in mm.
1.5*	0.25	+4	4.5±0.21	2.0±0.10
1.5	0.5	+4	4.4±0.16	1.9±0.18
1.5	0.7	+3	3.5±0.20	1.9±0.20
1.5	0.9	+3	3.6±0.21	2.2±0.11
1.5	1.1	+2	2.9±0.08	2.1±0.15
1.5	1.2	+1	2.5±0.10	2.2±0.17
1.5	1.5	0	1.8±0.12	1.8±0.11
0.0	1.5	0	1.9±0.13	1.8±0.11

* Three rats per experimental group.
Daily dose of ACTH divided into 3 equal injections, 8 hours apart. One mg. ACTH equivalent to 4 LA-1A units of Armour standard preparation.

reduced 25% by 0.7 mg. ACTH daily, and with increased dosage complete inhibition occurred at 1.5 mg. per day. It also was observed that the inhibitory effect of 1.5 mg. ACTH was as pronounced when given the last two days of the injection period as when administered on all three. This dosage of ACTH when given to castrated pseudopregnant controls did not produce growth of the reproductive tract. Also, hypophysectomized sexually immature rats given 1 or 2 mg. ACTHdaily for four days and killed on the fifth did not show stimulation of the ovaries, uterus, or vagina. From this it seems that the secretion of adrenal cortical hormones in response to ACTH offers the most plausible explanation for the inhibition of progesterone in these experiments.

DISCUSSION

In a previous paper on the inhibition of decidual development by estrogens it was mentioned that their effectiveness was in general proportional to their estrogenic potency (Velardo and Hisaw, 1951). The compounds tested for progesterone inhibition in the present investigation differ widely with respect to the reaction produced by their administration and some are quite inactive. There is apparently no one thing with which ability to antagonize progesterone can be correlated. Pregnanediol, an inactive steroid, has a strong inhibitory effect on progesterone-induced decidual development while pregnanedione, which is equally inactive, produces

no inhibition even at high doses. 11-desoxycorticosterone acetate (DCA) will produce a progestational reaction at high doses while cortisone (cortone acetate) apparently will not, yet DCA is a stronger antagonist of progesterone in the decidual reaction than cortone acetate. The mechanism of such inhibitory reactions is of course at present quite obscure. There probably is a physiological peculiarity that a compound must be able to arouse in order to act as an inhibitor, and it is equally probable that a certain molecular structure is required to make this possible. A comparative study of a larger number of steroid compounds would be required to decide these points.

These observations on the inhibition of progesterone suggest that many interactions of a similar nature involving both physiologically active and inactive steroids may be possible. This thought has been expressed in one form or another by several investigators and has been ably discussed by Hertz and Tullner (1950). Enough is known regarding these reactions to make it certain that several different processes are involved. For our purposes here it is probably sufficient merely to point out that under certain conditions estrogen inhibits progesterone, in other situations it is the reverse, and yet in others they may take part in a synergistic reaction. It also is interesting when one considers a so-called physiologically inactive steroid. The one we know the most about is pregnanediol. This compound is a strong antagonist of progesterone in decidual growth but so are estrogens, testosterone, 11-desoxycorticosterone, and certain other steroids. Yet pregnanediol will not inhibit the action of estrogen on the uterus and vagina in castrated rats, nor antagonize the action of testosterone on the seminal vesicles and prostate, but it will block the protection DCA gives to suprarenalectomized rats exposed to cold. From this we may gather that the organ that responds is as important in these reactions as the compounds administered.

The competitive action of steroids may offer an explanation for the interruption of pregnancy resulting from the injection of steroid hormones that probably under normal conditions take no direct part in the physiology of the uterus. Burdick and Emerson (1939) report that 0.25 or 5.0 mg. of testosterone propionate daily, beginning on the day of mating, will interrupt pregnancy in mice, and that 2 mg. DCA under the same conditions give similar results (Burdick and Konanz, 1941). Courrier and Colonge (1951) found that 25 mg. cortisone acetate daily will produce resorption and abortion of young in rabbits. Seifter, Christian, and Ehrich (1951) report similar effects of cortisone in pregnant rats and mention that the maternal structures of the placenta were strongly affected. Pregnenolone was also effective and its action involved both fetal and maternal parts of the placenta. This effect of pregnenolone is of interest as

it did not inhibit progesterone in the growth of deciduomata and in fact it alone at 12 mg. daily gave a +2 reaction. In this connection it might be mentioned that pregnanediol will also interrupt pregnancy in rats and while its inhibition of progesterone offers a plausible explanation, there are other effects that may contribute to the end result. For instance, pregnanediol when given to an intact rat produces, among other effects, a decrease in weight of the ovaries, uterus, adrenals, and thymus, and reduces the length of survival in the cold by about half. These observations suggest that the inhibitory effects of one steroid upon the action of another may not be due entirely to competition for metabolic control of the tissue or organ observed but that such effects may also be influenced by other reactions evoked elsewhere in the body by the competing compounds.

SUMMARY

Seven steroid compounds were tested for ability to inhibit progesterone in the development of deciduomata in castrated pseudopregnant rats. Young adult rats were castrated on the fifth day of pseudopregnancy, one horn of the uterus traumatized, 1.5 mg. progesterone given daily for three days, and the animals killed at the end of 72 hours. Deciduomata produced by such procedure were approximately the size of those formed in intact animals 72 hours after traumatization of the uterus on the fifth day of pseudopregnancy. The compounds tested were injected in graded doses concurrently with the standard daily dose of 1.5 mg. progesterone. Inhibition, when it occurred, was shown by a decrease in the diameter of the deciduomata as compared with the unopposed reaction to 1.5 mg. of progesterone. Compounds that produced inhibition did so in proportion to dosage. The daily dose required of the following compounds for complete inhibition of 1.5 mg. progesterone was as follows: pregnanediol 0.25 mg., testosterone 1.5 mg., 11-desoxycorticosterone 3.0 mg., and cortisone 4.5 mg. Pregneninolone produced incomplete inhibition and alone at doses of 12 mg. daily elicited small deciduomata. Pregnanedione and pregnenolone did not inhibit at 12 mg. daily and only the latter induced a decidual response when given alone. It may also be mentioned that ACTH in doses of 1.5 mg. daily caused complete inhibition. What determines the ability of these compounds to inhibit progesterone is not clear, and the nature of the reaction is equally uncertain. Competitive inhibition in the growth processes of decidual tissue offers a facile explanation. However, this probably is an over simplification as for example pregnanediol, though considered physiologically inactive when given alone, is capable of influencing several other endocrine processes which may tend to modify the end result.

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OXIDATION PRODUCT OF STILBESTROL

EFFECTS UPON THE RAT PITUITARY OF A NON-ESTROGENIC OXIDATION PRODUCT OF DIETHYLSTILBESTROL

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ON THE basis of various lines of evidence accumulated in the study of estrogen metabolism in women and in rodents, Smith and Smith (1941, 1946, 1948) have postulated that estrogenically inactive oxidation products of the estrogens, rather than estrogens *per se*, are directly responsible for the release of pituitary tropic factors. One line of evidence for this concept lay in the finding (Smith, 1944) that the presence of the testes or the simultaneous administration of progesterone to the castrate prevented the release of adrenotropic and gonadotropic factors from the pituitary following estrone administration to mature male rats. Progesterone, testosterone and adrenal cortical extract, in sufficient dosages, have been found to suppress the *in vivo* inactivation of the estrogens (see Smith, 1944, footnote 2, for references, and Segaloff, 1947). The observations in estrone treated rats, therefore, were interpreted as further support for the hypothesis that estrone increased the output of pituitary adrenotropic and gonadotropic factors through the *in vivo* formation of estrogenically inactive metabolites. That progesterone inhibits the effect of estrogens in causing release of pituitary gonadotropes in the female rodent has been reported by Phillips (1937), Makepeace *et al.* (1937), Astwood and Fevold (1939), Selye (1939), Bradbury (1947) and Lipschutz *et al.* (1948). A similar inhibitory effect of testosterone has been observed by Wolfe and Hamilton (1937), Segaloff and Dunning (1945) and Bradbury (1947). Lipschutz *et al.* (1948) have reported that desoxycorticosterone as well as progesterone inhibit the luteinizing effect that estradiol exerts upon intrasplenic ovarian transplants in guinea pigs. That the neutral steroids inhibit this particular response to the naturally occurring estrogens, therefore, appears to be well established.

In investigating the effects of diethylstilbestrol upon the mature male

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rat pituitary, Smith and Smith (1944) found that the release of tropic hormones following the administration of this synthetic estrogen was not inhibited by the presence of the testes or the simultaneous administration of progesterone. This suggested either that the pituitary stimulating effect, in the case of stilbestrol, was a direct response to the intact estrogenic molecule or that its inactivation was not inhibited by the neutral steroids. In subsequent observations, to be reported herein, there was evidence that release of pituitary tropins from stilbestrol, like that from estrone, was unrelated to its estrogenic potency but mediated by some estrogenically inactive product. These initial observations were the basis for the present investigation, the object of which was to isolate inactivation products of stilbestrol in the hope that some estrogenically inactive compound might be found that behaved like its parent substance in causing release of pituitary tropic factors. Our reason for searching for a non-estrogenic compound with this property was to avoid, in it clinical use, the pituitary inhibiting effect of the estrogens, an effect which, according to our findings to date, appears to be a response to the intact estrogenic molecule. Such a compound might be of considerable therapeutic value, particularly in problems in infertility related to inadequate luteal function of the ovary.

Preliminary experiments with dilute weakly alkaline solutions of stilbestrol that had lost estrogenic activity. It was reported by Zondek *et al.* (1943) that

TABLE 1. DEMONSTRATING THE ROUTINE EXPERIMENTAL PROCEDURE IN TESTS UPON MATURE MALE RATS (LITTERMATE VERSUS LITTERMATE)

Intact males—70 to 100 day old and weighing 150 to 200 gm. at start

Rat	Litter No.	Age at start, days	Wt. at start, gm.	Injections (1.0 cc. s.c. A.M. and P.M. for 5 days)	Autopsy on morning of 6th day						
					Weight of			Change vs. littermate control			
								Adrenals		Pituitary	
					Rat, gm.	Adr., mg.	Pit., mg.	mg.	%	mg.	%
A	1	79	190	None	196	25.1	5.50				
B	2	81	195	(controls)	202	31.2	6.51				
C	1	79	195	0.001 N NaOH	200	25.6	5.62	+0.5	+ 2.0	+0.12	+1.1
D	2	81	191	(10 cc./5 days)	200	29.9	6.22	−1.3	− 1.7	−0.29	−4.4
E	1	79	192	Stilbestrol in 0.001 N NaOH*	198	30.6	6.65	+5.5	+21.9	+1.15	+20.9
F	2	81	195	(10 cc./5 days)	205	38.2	8.24	+7.0	+22.4	+1.73	+26.5

Pituitary recipients—19- to 25-day-old female rats weighing 25 to 40 gm. at start. Each received 2 pituitaries (from above males) suspended in 6 cc. normal saline and injected in 6 injections over 3 days

Litter No.	At start		Pituitary donors (above males)	Autopsy on morning of 5th day						
				Weight of			Change vs. littermate control			
							Adrenals		Ovaries	
	Age, days	Wt., gm.		Rat, gm.	Adr., mg.	Ov., mg.	mg.	%	mg.	%
3	20	28.0	A plus B (controls)	35.0	11.6	51.0				
3	20	27.5	C+D (vehicle only)	35.0	12.0	48.7	+0.4	+ 3.5	− 2.3	− 4.5
3	20	29.0	E+F (test material)	37.5	9.4	36.2	−2.2	−19.1	−14.8	−29.0

* Freshly made up to contain 1 μg. per cc.

dilute alkaline solutions of diethylstilbestrol left at room temperature lost all estrogenic activity. In confirmation of this, solutions of stilbestrol were made up to contain 1.0 μg. per cc. of 0.001 N NaOH. Their estrogenic potency when first made up was 1 R.U. to 0.15 μg. of stilbestrol. After standing at room temperature for two weeks they were found to be non-estrogenic in 6 μg. dosages and ether extracts of the acidified solutions in olive oil were non-estrogenic in amounts equivalent to 20 μg. The effect of these solutions upon the pituitaries of mature male rates was tested as follows:

Procedure for tests on mature male rats (see Table 1). Ten cc. of these inactive aqueous solutions were injected in 10 divided dosages over 5 days into each of 6 mature male rats, each with 2 littermate controls of the same weight (±5 gm.), one of which received no injections and the other of which was injected with 10 cc. of 0.001 N NaOH. On the sixth day all 18 animals were killed and their pituitaries and adrenals weighed on a torsion balance. The pituitaries were ground in a small mortar and quantitatively transferred through a 22-gauge needle with 6 cc. of normal saline to a 15-cc. centrifuge tube for injection into immature female rats. These suspensions were mixed well before each injection and refrigerated throughout the injection period. Two pituitaries from experimental animals were injected in 6 divided doses over 3 days into each of 3 immature female rats, each with 2 littermate controls of the same weight (±1.0 gm.), one of which received 2 pituitaries from uninjected donors and the other of which received 2 pituitaries from animals that had been injected with 0.001 N NaOH only. All 9 rats were killed on the fifth day and their ovaries and adrenals weighed.

The results, presented in Table 2, were entirely similar to those previously reported by Smith and Smith when total dosages of 5 to 50 μg. of active stilbestrol were similarly tested (1944). A total dosage of 1 μg. of stilbestrol has no effect on the organ weights of the injected animals or the pituitary recipients in this test (Smith and Smith, 1944). The inactivated material, according to its estrogenic potency, contained certainly less than 0.25 μg. and probably less then 0.075 μg. of unchanged stilbestrol

TABLE 2.* EFFECT OF INACTIVATED DIETHYLSTILBESTROL (14 DAYS AT ROOM TEMPERATURE IN 0.001 NaOH) UPON THE PITUITARIES AND ADRENALS OF MATURE MALE RATS AND UPON THE TRANSPLANTABLE ACTIVITY OF THE PITUITARIES

Treatment of male donors	Male donors			Pituitary recipients†	
	No. rats	Adrenals	Pituitaries	Adrenals	Ovaries
Uninjected contr.	6	28.7 mg. (ave.)	6.31 mg. (ave.)	12.5 mg. (ave.)	51.1 mg. (ave.)
		Percentage change versus uninjected littermate			
Inactiv. Stilb. 10 μg./5 days	6	+26.0±7.8	+20.8±5.9	−18.7±7.6	+28.6±7.4
p		<0.01	<0.01	0.05	0.01
Vehicle contr.	6	+ 0.4±3.7	+ 0.3±3.8	+ 1.8±3.3	+ 1.7±5.6

* In this and in the following tables the ± figures after the average values for percentage change refer to σ or the standard deviation ($\sqrt{\Sigma d^2/(n-1)}$). From this, t (average change observed $\div \sigma/\sqrt{n}$) was calculated and p (or probability value) is from the Table of t, page 169; in Fisher's "Statistical Methods for Research Workers," ninth edition.

† The number of recipients is always one-half the number of donors.

in 10 cc. of solution, the dosage level at which these tests were run. The release of pituitary adrenotropic and gonadotropic factors observed, therefore, would appear to have been stimulated by some non-estrogenic breakdown product.

Crystalline products from alkali inactivated stilbestrol. Three crystalline products have been recovered by one of us (R.E.V.) from solutions of stilbestrol oxygenated in alkaline solution. One of these was isodienestrol, the identification of which from such solutions has already been reported by Vanderlinde and Westerfeld (1949). This compound was prepared from solutions in which 3 to 5 gm. of stilbestrol were left either at 37° C. or at room temperature for two weeks, bubbling in O_2 every other day. The starting concentrations were 2 mg. of stilbestrol per cc. 0.02 N NaOH, considerably higher than had been used in the experiments described above. In one preparation in which the conditions were exactly as in the original experiments, that is, standing at room temperature for two weeks at a concentration of 1 μg. per cc. 0.001 N NaOH, no crystalline products were recovered from 0.800 gm. of stilbestrol. In another experiment, however, in which 2.4 gm. of stilbestrol were inactivated for two weeks at room temperature with oxygenation at a concentration of 3 μg. per cc. 0.001 N NaOH, two compounds were recovered in the form of white and yellow crystals, the former melting at 239 to 243° C. and the latter at 250 to 253° C. These were mechanically separated and acetylated, yielding 6.5 mg. of white crystals melting at 156 to 157° C. and 3 mg. of yellow crystals melting at 83 to 84° C. A second similar experiment yielded an additional 69 mg. of the 157° C. acetate and none of the 84° C. acetate. There was too little of the yellow 84° C. acetate for further characterization, and since, in the biological tests to be reported below it showed no interesting properties, it will not be pursued further.

The final identification of the 157° C. acetate will be reported separately (by R. E. Vanderlinde and W. W. Westerfeld) when accomplished. Its C-H analysis agrees with a $C_{22}H_{20}O_8$ formulation and it appears to be a carboxylic acid. The absorption spectrum of the acetate in ethyl alchohol at 17.6 μg. per cc. showed a maximum at 250 mu. with a molecular extinction coefficient of 25,900. The free phenol, melting point 242 to 243° C., at an equivalent molar concentration, showed a maximum at 281 mu. with a molecular extinction coefficient of 24,000. Isodienestrol may be an intermediate in the formation of this new, highly oxygenated inactivation product of stilbestrol.

Biological testing of inactivation products of stilbestrol.

Methods. Three biological methods of testing the influence upon the rat pituitary were employed (Smith, 1944, 1945). The first of these is upon the *intact mature male rat* following the procedure outlined above and illustrated in detail in Table 1. Our reason for com-

paring littermates of the same weight (±5 gm.) rather than miscellaneous rats of the same age and weight is apparent in the data given in Table 1. Rats A and B, both uninjected, were of approximately the same age and weight at the start of the experiment, but they were not littermates and the adrenals and pituitary of B were 20% heavier than those of A. Rat C, on the other hand, the littermate of A that received the vehicle only, had adrenals and pituitary weighing only 2% more than those of A. By using *littermates*, therefore, and *expressing results in percentage figures*, animal variability is greatly reduced and one may uncover significant changes with a relatively small number of animals (*e.g.*, see Table 2). It has been found that strict limitations in the age and weight of the animals are also necessary for consistent results. They must have reached sexual maturity, be still growing and not over 210 gm. in weight at the start of the experiment. In our colony positive results are the most marked when the control pituitaries at autopsy weigh 6 to 8 mg.

The second test is upon intact 19- to 25-day-old female rats for the *enhancement of FSH*. A minimal effective dose of a follicle stimulating preparation is given in 6 divided dosages over 3 days to an immature female rat. To a littermate of the same weight (±1 gm.) the same dosage of FSH is similarly injected, but on the third, fourth and fifth days the test material is also injected; in 6 injections if in aqueous medium and in 3 injections if in oil. Vaginal smears are taken on both rats on the fifth and sixth days and both are autopsied on the morning of the seventh day. The ovaries are examined and weighed and *results are expressed in terms of the percentage change in weight of ovaries of the experimental rat as compared with those of its littermate control that received FSH only*. The dosage of FSH given should be the smallest amount that will produce estrous smears in 19- to 25-day-old intact females. The preparation of FSH used should be sufficiently pure so that twice this dosage gives no luteinization of the ovaries at autopsy on the 7th day. We now routinely employ Schering's sheep pituitary extract 2SY1.

The third test is for *inhibition of the castration changes of the pituitary* in female rats spayed at 30 to 45 days of age. Two of 3 littermates of the same weight (±2 gm.) are ovariectomized. To one of the spayed animals the test material is given in daily injections over the following 16 days. On the seventeenth day all 3 are autopsied and their adrenals and pituitaries weighed. The pituitaries are assayed as in the mature male rat test (*v.s.* and Table 1), 2 pituitaries from similarly treated rats being used for each assay upon littermate immature female rats. *All results are expressed in terms of percentage change as compared with intact uninjected littermates.* By the sixteenth day after ovariectomy the untreated females usually show significant increases in adrenal and pituitary weights as compared with intact littermates and always show very marked increases in the transplantable gonadotropic activity of the pituitary, particularly in its follicle stimulating effect as judged by the ovaries of the recipients. The above procedure provides a test for inhibition of these castration changes, complete inhibition being evidenced by organ weights in the injected animals and in their pituitary recipients that do not differ significantly from those of the intact littermates.

Certain compounds closely related to stilbestrol as well as the products obtained from alkali inactivation were assayed for their estrogenic potency (adult spayed female rats) and subjected to the above tests for their influence upon the pituitary. The compounds investigated are listed in Table 3 with their comparative estrogenic potencies. Detailed results on the pituitary responses will be given only for the 157° C. acetate, since

TABLE 3. SYNTHETIC COMPOUNDS* RELATED TO STILBESTROL WHOSE INFLUENCE UPON RAT PITUITARIES WAS INVESTIGATED. MELTING POINTS AND ESTROGENIC ACTIVITY

Compound	Free phenol		Diacetate	
	m.p.	1 R.U.	m.p.	1 R.U.
Stilbestrol	168–169° C.	0.15 μg.	—	—
Iso-dienestrol	185° C.	90.0 μg.	147–148° C.	>500 μg.
Dienestrol	228° C.	0.25 μg.	—	—
Gamma-dienestrol	—	—	75–76° C.	>100 μg.
Indenestrol "A"	173.5–175° C.	0.6 μg.	—	—
Unidentified products of alkali inactivation				
157° C. acetate	240–243° C.	>100 μg.	156–157° C.	>100 μg.
84° C. acetate	—	—	84° C.	> 60 μg.

* The crystalline stilbestrol used in preparing its derivatives was generously supplied by E. R. Squibb and Sons, through Dr. H. Sidney Newcomer, and by Ayerst, McKenna and Harrison, Ltd., through Dr. E. C. Reifenstein. Indenestrol "A" and gamma dienestrol diacetate were obtained through the courtesy of Dr. C. W. Sondern of White Laboratories, Newark, N. J.

this was the only one of those listed that behaved like our original solutions of dilute alkali inactivated stilbestrol, that is, caused increased secretion and release of adrenotropic and gonadotropic factors but did not inhibit the post-castration increase in pituitary activity.[1] A brief summary of the findings on the others follows.

Dienestrol and Indenestrol "A", both of which are estrogenic, caused adrenal and pituitary hypertrophy in the adult male rat test in total dosages of 50 μg. and enhanced the ovarian response of immature female rats to FSH in 20 μg. amounts. They also, however, like stilbestrol itself but unlike the alkali inactivated solutions (*v.i.*) inhibited the post-castration increase in transplantable gonadotropic activity of the pituitary in 2 to 10 μg. dosages. Dienestrol was more potent in this respect than indenestrol "A", as might be expected considering its greater estrogenic activity. *Gamma-dienestrol* diacetate and the *84° C. acetate* were estrogenically inactive and did not inhibit the castration change in 20 μg. dosages. They were also found, however, to be inactive as pituitary stimulants both in the mature male rat test when 50 μg. were given and in the FSH enhancement test in 20 μg. amounts. *Iso-dienestrol*, being a non-estrogenic product of alkali treatment, was extensively investigated in all of these tests for pituitary effects. The responses to the free phenol as well as to the diacetate differed with different dosages.

[1] We have omitted from the list in Table 3 a compound with biological properties entirely similar to those of the 157° C. acetate. This is the monobenzyl ether of stilbestrol, a sample of which was given to us in 1948 by Dr. Nicholas Werthessen of the Worcester Foundation for Experimental Biology. According to our tests, it is very low in estrogenic potency, very potent as a pituitary stimulant and does not inhibit the post-castration increase in FSH activity of the pituitary, in fact, appears to enhance the luteinizing response in this test. Dr. Werthessen (personal communication) had done a great deal of experimental work with this compound prior to ours and is now subjecting it to clinical trials. The initial reports on its biological properties and clinical value should come from him.

Ten to 50 μg. gave no evidence of stimulation of the pituitaries of mature male rats or of those of immature females given FSH, whereas 100 μg. dosages caused questionably significant enlargement of the adrenals and pituitaries of mature males. Total dosages of 2 to 50 μg. partially inhibited the castration increase in pituitary gonadotropic potency but 50 μg. were less effective in this respect than 25 μg. Such inconsistencies might theoretically reflect *in vivo* conversion of the iso-dienestrol to the free phenol of the 157° C. acetate if enough is administered to the intact animal and to some more estrogenic product in the castrate. In any event, this compound showed no evidence of having desirable properties from the clinical point of view.

TABLE 4. EFFECT OF THE 157° C. ACETATE UPON THE PITUITARIES, ADRENALS AND SEMINAL VESICLES OF MATURE MALE RATS AND UPON THE TRANSPLANTABLE ACTIVITY OF THE PITUITARIES

Treatment of male donors	Mature male pituitary donors				Pituitary recipients*	
	No. rats	Adrenals	Pituitaries	Sem. ves.	Adrenals	Ovaries
Uninjected contr.	18	27.5 mg. (ave.)	6.87 mg. (ave.)	235 mg. (ave.)	13.4	83.1
		Percentage change versus uninjected littermate				
157° C. acetate (50 μg./5 days)	18	+35.1±21	+20.7±12	+42.0±28	−2.8 ±17	−10.5±13
P		<0.01	<0.01	<0.01		0.05
Vehicle contr.	27	+ 1.3± 5.5	+ 1.1± 3.9	− 3.0±22	+0.97± 4.8	+ 0.73± 6.3

* The number of recipients is always one-half the number of donors.

Results with the 157° C. acetate

Mature male rat test. Table 4. In this test the non-estrogenic 157° C. acetate behaved like stilbestrol (Smith and Smith, 1944) in that it caused an increase in adrenal and pituitary weights. That the adrenal hypertrophy reflected increased cortical secretion was indicated by the cytological findings shown in Figure 1 and for which the authors are greatly indebted to Dr. Roy O. Greep. Dr. Greep found the same cytological differences between the adrenals of treated and control animals in a large series of sections that he prepared and examined for us. That the pituitary hypertrophy reflected increased gonadotropic as well as adrenotropic secretion was indicated by the increased weight of the seminal vesicles. We have not observed any significant increases in the weights of seminal vesicles in tests upon estrogens or upon related pituitary stimulating compounds that have been investigated.[2] The other compounds tested, however, all

[2] The pituitary stimulating properties of Dr. Westerfeld's preparation of estronelactone (1942) were reported upon by Smith in 1944 and 1945, together with the comment that not all preparations of this compound were active in this respect, an observation also made by Bradbury (1947). Since that time, various preparations of estronelactone have been tested in this laboratory through the kindness of Dr. Westerfeld, Dr. R. W. Bates of E. R. Squibb and Sons, Dr. Robert R. Levin of the Upjohn Company, Dr. Alan Mather of Clark University, Dr. Erwin Schwenk of the Schering Corporation

had some estrogenic potency and it is well known that estrogens inhibit the male gonads, presumably through inhibition of FSH. The complete lack of estrogenic activity in the 157° C. acetate, therefore, may account for the clear-cut hypertrophy of the seminal vesicles in these experiments, a response to the combined action of uninhibited FSH plus enhanced LH that may have been masked when estrogen was administered and the normal production of FSH thereby inhibited.

The absence of estrogenic activity in the 157° C. acetate may also account for the fact that the pituitaries failed to show the marked decreases in transplantable gonadotropic and adrenotropic activity previously seen in donors treated with estrogenic compounds. It has been generally accepted that this decrease in potency of the pituitary reflects increased release of the tropic hormones so that the gland itself contained less. It seems quite possible that the complete absence of any estrogenic potency renders the 157° C. acetate a more effective agent than the estrogens, so that increased secretion as well as release of pituitary tropic factors result from its administration, and the glands themselves, at the time of autopsy, reflect a combination of these effects.

Enhancement of FSH. Table 5. From the results presented it is apparent

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and Dr. R. P. Jacobsen of the Worcester Foundation for Experimental Biology. The acetates as well as the free phenols were tested. Some of these preparations were found to give the same evidence of pituitary stimulation with no inhibition of castration changes as was originally reported, whereas others failed to exhibit these properties. Those that exhibited them all had some slight estrogenic activity (1 R.U./10 to 20 μg.) but the estrogenic potency bore no constant relationship to the biological response, some of the more estrogenic of the preparations being completely ineffective as pituitary stimulants. Furthermore, the acetates of the effective preparations were always considerably less estrogenic than the free phenols, but fully as active in causing release of pituitary tropic factors. Jacobsen's "estrololactone" (1948) and its derivatives were extensively investigated, since this appeared to be the purest preparation available of estrone-lactone. According to infra-red spectography, it and Westerfeld's estrone-lactone are identical. Its estrogenic activity was very low (1 R.U./100 μg.). It had no effect on the weights of the adrenals, pituitaries or seminal vesicles of mature male rats but increased the gonadotropic potency of their pituitaries. It did not enhance the response of immature rat ovaries to FSH, but did enhance the increase in gonadotropic potency of the pituitaries that follows ovariectomy, particularly as regards follicle stimulating activity. The response of the rat pituitary, in other words, appeared to be one of increased storage of FSH, rather than release of LH, as is indicated by the findings with Dr. Westerfeld's preparations. It was thought that the effects of the latter might be due to contamination with small amounts of unchanged estrone, but the addition of estrone to Jacobsen's estrololactone in amounts that brought its estrogenic activity up to that of Westerfeld's preparation did not change the response of mature male rats. It was concluded that the pituitary stimulating properties of Westerfeld's preparation (and the others that behaved similarly in these tests) must be due to contamination with some other non-estrogenic product of estrone oxidation rather than to estrone-lactone itself.

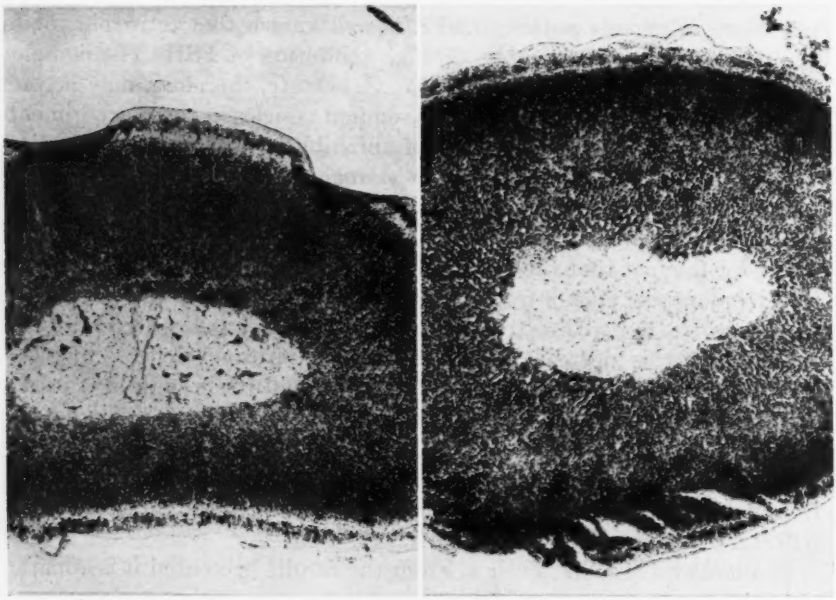

FIG. 1. *Left*: Adrenal of intact mature male rat; untreated. Transition zone present. Frozen section; Sudan IV. *Right*: Adrenal of intact mature male rat after 50 μg. 157° C. acetate in 5 days. Frozen section; Sudan IV. Note absence of transition zone, increased sudanophilia in outer fasciculata and increased width of the cortex.

that the 157° C. acetate, even more markedly than its estrogenic parent, enhanced the growth promoting effect of a follicle stimulating preparation upon the ovaries of immature female rats.[3] In fact, our results in this test

TABLE 5. ENHANCEMENT OF FSH WITH DIETHYLSTILBESTROL AND WITH THE 157° C. ACETATE

Injections to immature female rats	No. of rats	Effect on ovaries	
		Av. wt. mg.	Percentage change versus littermate injected with FSH only
FSH only	47	14.3	
FSH plus vehicle	25	14.1	−1.28 ± 8.7
FSH plus stilbestrol 20 μg./3 days	20	17.4	+21.5 ± 38 (P = 0.02)
FSH plus 157° C. acetate 20 μg./3 days	21	21.5	+50.4 ± 31 (P = <0.01)

[3] In earlier tests upon Westerfeld's estrone-lactone for the enhancement of FSH an increased number of grossly visible discrete corpora lutea was noted in the experimental animals (Smith, 1945). In the results presented in Table 5 there were no discrete corpora lutea either in the 72 controls or the 41 animals that received stilbestrol or the 157° C.

have been more clear-cut with the 157° C. acetate than with preparations of estrone inactivation products,[2] all of which had some estrogenic activity. Here again one is tempted to postulate that the presence of estrongenic activity partially counteracts the pituitary stimulating property of an otherwise active compound by inhibiting endogenous FSH.

Effect on castration changes of the pituitaries. That estrogens inhibit the cytological changes and the increased gonadotropic potency of the pituitary that follows castration is well established. In Table 6 are our results when 1 to 10 μg. of stilbestrol were given to immature female rats during the

TABLE 6. EFFECT OF DIETHYLSTILBESTROL ON CASTRATION CHANGES OF THE PITUITARIES OF IMMATURE FEMALE RATS (SPAYED AT 30 DAYS OF AGE)

Treatment of donors	Immature female pituitary donors			Pituitary recipients*	
	No. rats	Adrenals	Pituitaries	Adrenals	Ovaries
Intact controls (no injections)	12	20.2 mg. (ave.)	3.86 mg. (ave.)	10.6 mg. (ave.)	16.0 mg. (ave.)
		Percentage change versus intact littermate			
Spayed controls (no injections)	12	+18.8±10	+31.4±23	+15.5±3.7	+103 ±65
P		<0.01	<0.01	0.01	0.01
Spayed: stilbestrol 1 to 10 μg./16 days	12	+10.8±10	+34.8±30	+ 6.0±9	+ 23.3±37
P		<0.01	<0.01	0.2	0.2

* The number of recipients is always one-half the number of donors.

first 16 days after spraying, the operation having been performed in this group at 30 days of age. Adrenal and pituitary hypertrophy as well as increased adrenotropic and gonadotropic potency of the pituitary follow castration at 30 days of age in our animals. The increased gonadotropic potency is more marked when the animals are more mature at the time of spaying, while hypertrophy of the pituitaries is highly significant only when the animals are younger and when the intact controls have small pituitaries (3 to 5 mg.) at the time of autopsy. This is apparent by comparison of the two different age groups represented in Tables 6 and 7, respectively.

acetate. This is probably due to the fact that the sheep pituitary extract used in these experiments had no luteinizing effect *per se*, whereas in our earlier tests menopausal urine was used as the source of FSH and 8% of the control animals receiving this preparation developed luteinization of the ovaries apparent upon inspection. We now use only the sheep pituitary preparation since results with M.U., although more spectacular, are less reproducible because of the fact that no two extracts of menopausal urine behave in exactly the same manner. Despite the failure to produce corpora lutea in sheep pituitary FSH enhancement tests, the 50% increase in ovarian weights with the 157° C. acetate is highly significant and may probably be assumed to reflect LH release.

In subjecting estrone to this test (Smith, 1945) it was found that a total dose of 10 μg. over 16 days inhibited the castration hypertrophy and the cytological changes as well as the increased transplantable potency of the gland. Greep and Jones (1950) observed no increase in the size of the pituitaries in untreated female rats spayed at 30 days of age and autopsied 45 days later. They do record, however, a depression of pituitary weights in such animals when as little as 0.2 R.U. of estradiol benzoate was given daily during the 45 days after castration. With larger dosages of estrogen these investigators and others (Finerty and Meyer, 1950) observed either no effect on the size of the gland or a definite enlargement as the dosage

TABLE 7. EFFECT OF THE 157° C. ACETATE ON CASTRATION CHANGES OF THE PITUITARIES OF IMMATURE FEMALE RATS (SPAYED AT 45 DAYS OF AGE)

Treatment of donors	Immature female pituitary donors			Pituitary recipients*	
	No. rats	Adrenals	Pituitaries	Adrenals	Ovaries
Intact controls (no injections)	12	25.8 mg. (ave.)	6.78 mg. (ave.)	12.0 mg. (ave.)	13.5 mg. (ave.)
		Percentage change versus intact littermate			
Spayed controls (no injections)	12	+21.0±20	+ 8.6±14	+15.6±13	+278±105
P		<0.01	0.05	0.05	<0.01
Spayed 157° C. acetate 20 μg./days	12	+23.0±21	+10.5±10	+24.8±19	+310±130
P		<0.01	<0.01	0.02	<0.01

* The number of recipients is always one-half the number of donors.

was progressively increased towards the toxic level. Under the conditions of our experiments, stilbestrol, in total dosages of 1 to 10 μg., had no significant effect on the castration hypertrophy of the adrenals and pituitaries. Even in these relatively small dosages, stilbestrol was apparently toxic to the spayed rats, since their weight gains, over the 16 days of injections, were significantly less than those of their control littermates. The enlarged adrenals and pituitaries of the experimental animals may have been referable to this toxicity, thereby explaining the fact that stilbestrol, unlike estrone in small dosages (Smith, 1945), failed to exhibit any inhibitory effect upon castration hypertrophy. That the castration increase in the content of adrenotropic and gonadotropic factors was inhibited, however, was evidenced by the fact that the weights of the adrenals and ovaries of the recipients of pituitaries from stilbestrol treated castrates did not differ significantly from those of littermates that received pituitaries from intact control donors.

The 157° C. acetate, on the other hand, had no effect on any of the castration changes (Table 7). The experimental animals received a total of

20 μg. of this compound over the 16 days following spaying and showed essentially the same increase in adrenal and pituitary weights and in transplantable activity of the pituitaries as did their uninjected spayed littermates. This negative finding in conjunction with it pituitary stimulating effect as revealed by the other two tests is the reason for our special interest in this compound.

DISCUSSION

Until the 157° C. acetate shall have been chemically identified and some more practical means of preparing it devised, its biological properties as reported above will remain of more theoretical than practical value. It seems quite possible that clinical attempts to utilize the pituitary stimulating properties of the estrogens in non-pregnant women have met with very indifferent success because of a dual response to therapy such as is observed in the rat. A compound that retains the pituitary stimulating properties of an estrogen but has no inhibitory effect might well be of considerable therapeutic value.

That a non-estrogenic oxidation product of an estrogen is capable of causing release of pituitary adrenotropic and gonadotropic factors in the rat is of considerable theoretical interest. Its very lack of estrogenic potency, in fact, appears to make this compound more potent than its parent substance, stilbestrol, as a pituitary stimulant. It is also of interest that the ability of stilbestrol to inhibit the increase in pituitary gonadotropic potency that follows ovariectomy is entirely lacking in this non-estrogenic oxidation product. It should be emphasized that the test for inhibition of castration changes described in this communication involves autopsy 16 days after spaying, when the most pronounced change in the transplantable gonadotropic activity of the pituitary appears to be an increase in its follicle stimulating activity rather than in its content of luteinizing hormone. Certain non-estrogenic steroids such as testosterone are, like the estrogens, capable of reducing the gonadotropic potency of the pituitaries of male or female castrates (Greep and Jones, 1950), but only when comparatively large dosages are given. The estrogens, on the other hand, are effective as inhibitors of this castration change in very small dosages and, according to our findings to date, their potency in this respect appears to be directly proportional to their estrogenic activity. The findings presented are in keeping with the hypothesis that the pituitary is inhibited by the estrogens as such, that is, by the intact estrogenic molecule, whereas increased secretion and release of tropic factors are properties unrelated to their estrogenic activity and are exhibited by non-estrogenic metabolites produced during the process of oxidative inactivation.

SUMMARY

Dilute aqueous alkaline solutions of diethylstilbestrol lose estrogenic potency but retain their ability to cause the release of adrenotropic and gonadotropic factors from the pituitary of the rat.

From such solutions a crystalline compound has been recovered and partially characterized as a carboxylic acid, a highly oxygenated product whose acetate melts at 157° C.

This 157° C. acetate is non-estrogenic in spayed female rats in 100 μg. dosages, but retains the properties of its parent substance so far as pituitary release of tropic factors is concerned.

Unlike stilbestrol and other estrogens, the 157° C. acetate does not inhibit the increased adrenotropic and gonadotropic potency of the pituitary that follows ovariectomy.

The theoretical implications and the possible therapeutic value of a compound with such properties are considered.

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ASCORBIC ACID INTAKE AND THE ADRENAL CORTEX[1,2]

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THE isolation and demonstration of a high concentration of ascorbic acid in the adrenal cortex, by Szent-Györgyi (1928), has stimulated investigators for many years to ascertain whether or not there is any correlation between ascorbic acid and adrenocortical function. Quick (1933) postulated that the adrenal hypertrophy in pre-scorbutic and scorbutic animals previously observed by Bessessen (1923) was due to an attempt on the part of the adrenal gland to compensate for the deficiency of ascorbic acid. Mouriquand, Tete, and Lavaud (1938), and Harris and Ray (1933) studied the adrenals in chronic scurvy and reported a reduction in ascorbic acid concentration that did not parallel the clinical change in the animal. Giroud and coworkers (Giroud, Santa, Martinet and Bellon 1940) stated that ascorbic acid depletion of the adrenal cortex is accompanied by a definite decrease in adrenal cortical hormone production. Further they claimed that not only does ascorbic acid enhance the production of the hormones but it also improves their utilization. These observations, however, were not supported by biochemical or morphological data (Giroud, and Santa 1939; Giroud, Santa, Martinet, and Bellon 1940). Sayers and Sayers (1947) administered ACTH to normal and to hypophysectomized rats and demonstrated a rapid decrease in adrenal ascorbic acid and adrenal cholesterol. This was followed by a rapid return to normal levels of these substances in the rat, but at a much slower rate in the guinea pig. Adrenal hypertrophy with depletion of ascorbic acid was produced by prolonged administration of ACTH. Selye (1937) demonstrated that in various types of stress the adrenal gland enlarges, and subsequent workers (Sayers, Sayers, White, Fry, and Long 1944) demonstrat-

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ed that stress depletes the adrenal cortex of cholesterol and adrenal ascorbic acid (Torrance, 1940; Sayers, Liang, and Long, 1945; Fortier, Skelton, Constantinides, Timiras, Herlant, and Selye 1950). On the other hand, Dugal and Therien (1944) found that ascorbic acid in large doses not only prevented adrenal enlargement in cases of stress but in many instances caused a reduction in the size of the gland. Lowenstein, Zwemer and Pines (1940) have suggested that the adrenal cortex contains water-soluble steroids in which the steroid nucleus is associated with ascorbic acid. Further, Lowenstein and Zwemer (1946) in an unconfirmed report have claimed the isolation of an ascorbic acid conjugated compound with adrenocortical activity.

Although the evidence seems to favor the idea of a role of ascorbic acid in adrenocortical function, Long and other workers (Long 1947; Hyman, Ragan and Turner 1950) have suggested that the relationship between vitamin C and steroid production may not be very intimate. Long was led to this conclusion by the observation that administration of ACTH to scorbutic guinea pigs caused a fall in adrenal cholesterol and lymphocytes, although the ascorbic acid concentration in the adrenal was practically zero. These observations would suggest that ascorbic acid need not be present either in the adrenals or in the blood for the production and activity of cortical hormones. Vogt (1948) found no evidence to suggest that cortical hormones in adrenal blood was linked with ascorbic acid. In addition Daughaday *et al.* (Daughaday, Jaffe and Williams 1948) have shown that urinary excretion of cortical steroids is only slightly decreased in patients with scurvy. The entire problem has been reviewed recently by Sayers (1950).

Because of the unsettled state of this problem it seemed important to study the influence of various levels of ascorbic acid intake on the adrenal cortex and to measure morphologically and biochemically some of the factors known to be affected by adrenocortical activity.

MATERIAL AND METHODS

One hundred and twenty male guinea pigs obtained from a local dealer and weighing 250 to 300 grams, were adjusted to laboratory conditions for seven days on a complete pellet ration, free of ascorbic acid.* During this period of observation the guinea pigs were given orally 2.5 mg. ascorbic acid daily. At the end of the seven days the animals were individualized. They were then divided in groups according to weight, ascorbic acid intake and dietary regimen. Three groups of eighteen each were fed ad libitum, and the same number of animals was used as pair-fed controls. The experimental groups were given 0.0 (deficient), 0.5 mg. (partially deficient) and 2.5 mg. ascorbic acid daily (normal). Two of the pair-fed groups received 2.5 mg. and the third group 25 mg. ascorbic

* This diet was especially prepared by the Arcady Farms Milling Company of Chicago through the courtesy of Mr. F. J. Rech.

acid daily (fortified). An additional group of twelve animals was fed ad libitum and given 2.5 mg. ascorbic acid daily (controls). Tap water was used throughout the experiment. Diet consumption was measured daily. Weights were recorded every other day.

Prior to the beginning of each experimental period white blood cell counts, total eosinophil counts (Randolph, 1944), and differential smears were taken twice by ear puncture for baseline data. At seven, fourteen and twenty-one day intervals six animals from each experimental group with their six pair-fed controls were sacrificed under Nembutal anesthesia (30 mg./kg. body weight). One to two cc. of blood were taken by heart puncture in an heparinized bottle for whole blood ascorbic acid (Roe and Kuether 1943; Keuther, Telford, Roe 1944) and glucose determinations (Folin and Malmros 1929). The abdomen was opened and two liver samples were taken, one for cholesterol and the other in hot 35 per cent potassium hydroxide for glycogen (Good, Kramer and Somogyi 1933). The animal was then exsanguinated and the serum obtained was examined for cholesterol (Schoenheimer and Sperry 1943; Sperry and Bland 1943) and sodium (Consolazio and Dill 1941). The adrenals were rapidly removed and one-half of one adrenal was placed in a tared test tube containing 5.0 cc. of four per cent trichloroacetic acid for the determination of adrenal ascorbic acid (Roe and Kuether 1943). The second half of the adrenal was weighed on a Roller-Smith balance and fixed in ten per cent formalin stabilized with one per cent cadmium and calcium chlorides (Lillie 1949). The second adrenal was placed in a tared test tube and used for total and free cholesterol determinations. The total adrenal weight was obtained by the total of the two portions of the first adrenal plus the weight of the second adrenal. The liver was then removed and the total weight calculated from the sum of the samples plus the exsanguinated specimen. The thymus was inspected, the spleen weighed, and the pituitary removed and fixed in ten per cent formalin. Random sampling for histo-chemical determinations of adrenal ascorbic acid (Barnett and Bourne 1941; Deane and Morse 1948) was done by immediate fixation of thin slices in aqueous silver nitrate solutions.

An additional group of eleven male guinea pigs were fed ad lib.: six received no ascorbic acid, and five received 2.5 mg./day for 21 days. They were killed by exsanguination under Nembutal anesthesia to obtain data on dry weight, water, nitrogen and the total lipid content of the adrenals. The adrenals were removed immediately and weighed (wet weight). They were then placed in an oven at 100° C. for 24 hours under vacuum and weighed again at the end of this period to obtain dry weight and water content. Total lipids were obtained by differential weight after triple ether extraction of the dry adrenals (Eichelberger and Bibler, 1940). Nitrogen was determined chemically on the residue (Ma and Zuazaga, 1942).

Histological and histochemical studies were done on paraffin and frozen tissue sections. Sudanophilia was studied on frozen formalin-fixed tissue sections stained with either Oil Red 0 or Sudan IV counterstained with hematoxylin.

Birefringency was determined by using polarized light on unstained and Oil Red 0 stained tissue sections. The Schultz method was employed on all unstained frozen adrenal sections. The method is essentially an adaptation to histology of the Liebermann-Burchardt sterol reaction and has been considered rather specific for oxycholesterol, diols, or cholesterol and cholesterol esters when the concentration of cholesterol to other fats is above 1:10 (Everett 1947).

The specificity of other methods for the demonstration of steroids and ketosteroids in the adrenal cortex has been strongly criticized recently (Danielli, 1949; Sayers, 1950; Feldman 1950). These techniques, therefore, were not used in this study, with the exception of the procedure suggested by Ashbel and Seligman (1949) for the demonstration

of carbonyl groups which was applied to selected cases. The results obtained with this method, however, added little or no information to that which had been gained with Oil Red 0 and birefringence studies. A detailed discussion of the histochemical methods is reported elsewhere (Stepto, Pirani, Consolazio, Bell, and Marek 1951).

RESULTS

The clinical picture of ascorbic acid deficiency, as well as the body weight curves in this condition, are well known and need not be reported here.

Adrenal Weight (Table 1, Fig. 1). After seven days on the various levels of ascorbic acid intake there is little difference in the adrenal weight. At day 14 the adrenal weight is strikingly increased in the deficient animals (no ascorbic acid) and moderately increased in the partially deficient guinea

TABLE 1. ASCORBIC ACID INTAKE AND THE ADRENAL GLAND

Day	Ascorbic acid intake, mg./day	Adrenal wt., mg./100 gm. body weight	"t" (P)[a]	Adrenal ascorbic acid, mg./100 gm.	"t" (P)[a]	Adrenal cholesterol, mg./100 mg.	"t" (P)[a]
7	0.0	41± 3.4	0.85	11.3± 4.2	3.7	4.88±0.72	0.21
	2.5 pair fed	45± 3.4	(<0.4)	26.3± 1.1	(<0.01)	4.93±0.07	(>0.8)
	0.5	42± 4.4	0.50	23.3± 8.2	2.1	4.76±0.10	1.1
	2.5 pair fed	45± 4.4	(<0.6)	39.0± 1.4	(<0.05)	4.51±0.69	(>0.3)
	25.0	40± 2.0	0.83	54.1±15.0	1.3	5.60±0.23	1
	2.5 pair fed	42± 1.4	(<0.4)	34.1± 1.2	(0.2)	5.82±0.12	(>0.4)
14	0.0	74± 2.5	3.5	6.0± 0.3	3.7	2.52±0.14	11.6
	2.5 pair fed	48± 7.0	(<0.01)	32.0± 7.0	(<0.01)	4.97±0.53	(<0.001)
	0.5	57± 2.0	3.7	8.6± 2.1	3.6	4.73±0.70	2.8
	2.5 pair fed	42± 3.4	(<0.01)	24.0± 3.0	(0.01)	5.59±0.67	(0.02)
	25.0	44± 6.0	1.1	44.0±14.0	0.13	4.26±0.13	5.9
	2.5 pair fed	32± 4.0	(0.3)	46.0± 4.0	(0.9)	5.67±0.55	(<0.001)
21	0.0	93± 5.0	2.6	3.7± 0.1	39.4	2.84±0.16	14.6
	2.5 pair fed	64±12.0	(0.02)	43.1± 0.1	(<0.001)	5.92±0.14	(<0.001)
	0.5	52± 7.0	0	8.7± 1.1	12.9	4.79±0.21	3.9
	2.5 pair fed	52± 6.0		25.2± 0.8	(<0.001)	5.92±0.21	(>0.001)
	25.0	39± 3.0	0.75	48.5±16.0	0.15	4.54±0.15	5.9
	2.5 pair fed	42± 2.0	(<0.5)	46.0± 3.1	(<0.9)	5.85±0.16	(<0.001)

[a] P-values calculated according to Fisher table of values of t.

pigs (0.5 mg./day). Twenty-one days after the restriction of ascorbic acid, the adrenal weight of the deficient animals is markedly increased and there is a slight to moderate increase in adrenal weight of the pair-fed control animals. It should be noted that the deficient animals between 14 and 21 days had a marked drop in body weight accompanied by a decrease in diet intake. The adrenal hypertrophy thus is even greater when considered in relation to body weight. No significant differences are noted in the adrenal weight of the partially deficient and fortified (25 mg./day) animals as compared to their controls. Similarly no changes occur in the "normal" guniea pigs. Analyses (Table 2) of the dry adrenals of the deficient guinea pigs disclose a decrease in fat and an increase in protein in the hypertrophied adrenals as compared to those of the control animals. The

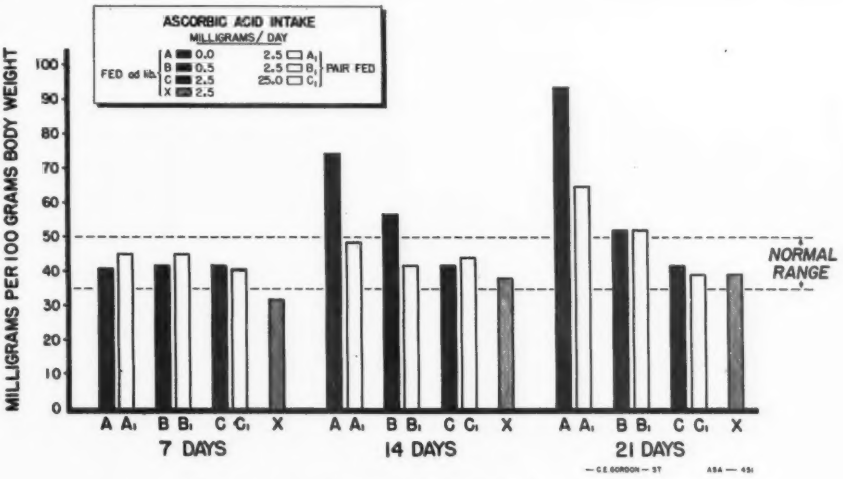


FIG. 1. Ascorbic acid intake and adrenal weight.

water content appears to be relatively the same for both the deficient and control animals.

Adrenal Ascorbic Acid (Table 1, Fig. 2). The adrenal ascorbic acid concentration is markely decreased after seven days of ascorbic acid-free

TABLE 2. ASCORBIC ACID INTAKE AND THE WATER, FAT AND PROTEIN CONTENT OF THE ADRENAL GLAND (21 DAYS)

No. animals	Ascorbic acid intake, mg./day	Adrenal wet wt.	Water %	Adrenal dry wt., mg.	Fat % dry wt.	Protein % dry wt.
6	0.0	221 ± 39.0	73.3 ± 2.0	57.3 ± 7	29.8 ± 0.6	31.8 ± 1.2
5	2.5	151 ± 5.0	74.7 ± 3.0	38.4 ± 8	52.9 ± 1.1	22.6 ± 0.8

regimen. The concentration is in the lower range of normal in the partially deficient animals. After 14 days a further decrease in adrenal ascorbic acid is noted in the deficient guinea pigs, while the partially deficient animals show only a moderate depletion. The pair-fed controls of both groups are within normal range. No differences are noted between the ascorbic acid values of the fortified and those of the normal (2.5 mg./day) animals.

Adrenal Cholesterol (Table 1, Fig. 3). The total adrenal cholesterol after seven days on the experimental levels of ascorbic acid intake is not significantly different. However after fourteen days the deficient guinea pigs show a severe progressive depletion of adrenal cholesterol. The pairfed controls have a steady rise in their adrenal cholesterol which parallels that of the normal animals. Large doses of ascorbic acid seem to have little

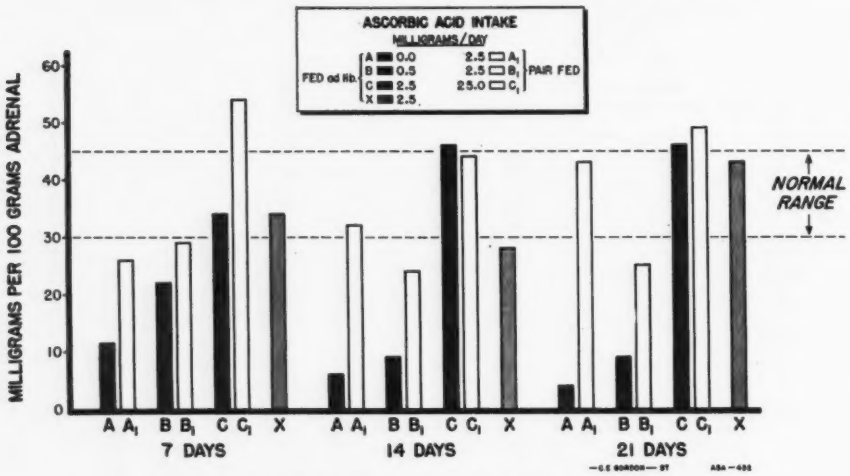


FIG. 2. Ascorbic acid intake and adrenal ascorbic acid.

effect on the adrenal cholesterol. From these data, then, it appears that withdrawal or markedly reduced intake of ascorbic acid is accompanied by a reduction in adrenal cholesterol. The reduction of adrenal cholesterol is, however, at a much slower rate than that of the adrenal ascorbic acid. The changes found in the cholesterol are principally in the ester fraction

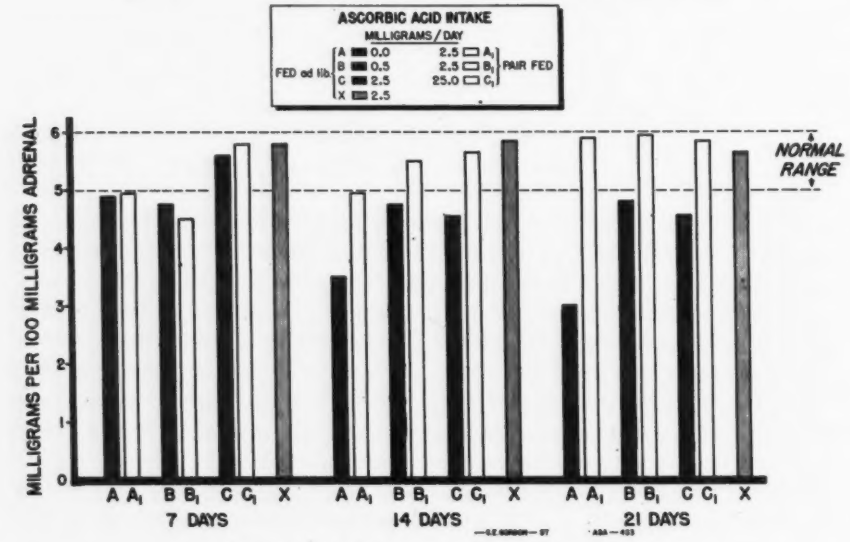


FIG. 3. Ascorbic acid intake and total adrenal cholesterol.

with the free fraction of the total cholesterol remaining relatively the same.

If the ratio of adrenal cholesterol per unit of adrenal ascorbic acid is calculated, it appears that there is an optimal value above which there are usually clinical signs of ascorbic acid deficiency (Fig. 4). This optimal ratio lies somewhere between 100 to 250 mg. of cholesterol per mg. of ascorbic acid. After seven days on the experimental diets, the ratio of adrenal cholesterol to adrenal ascorbic acid is high in the deficient animals and relatively low in animals on the other ascorbic acid regimen. At four-

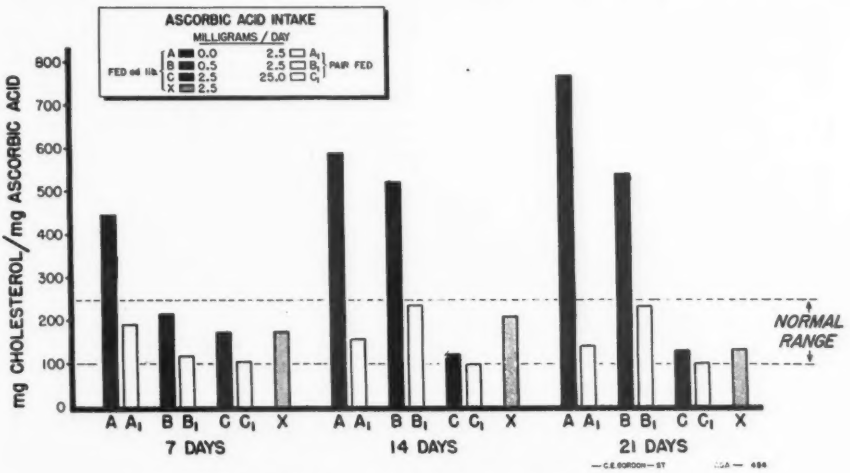
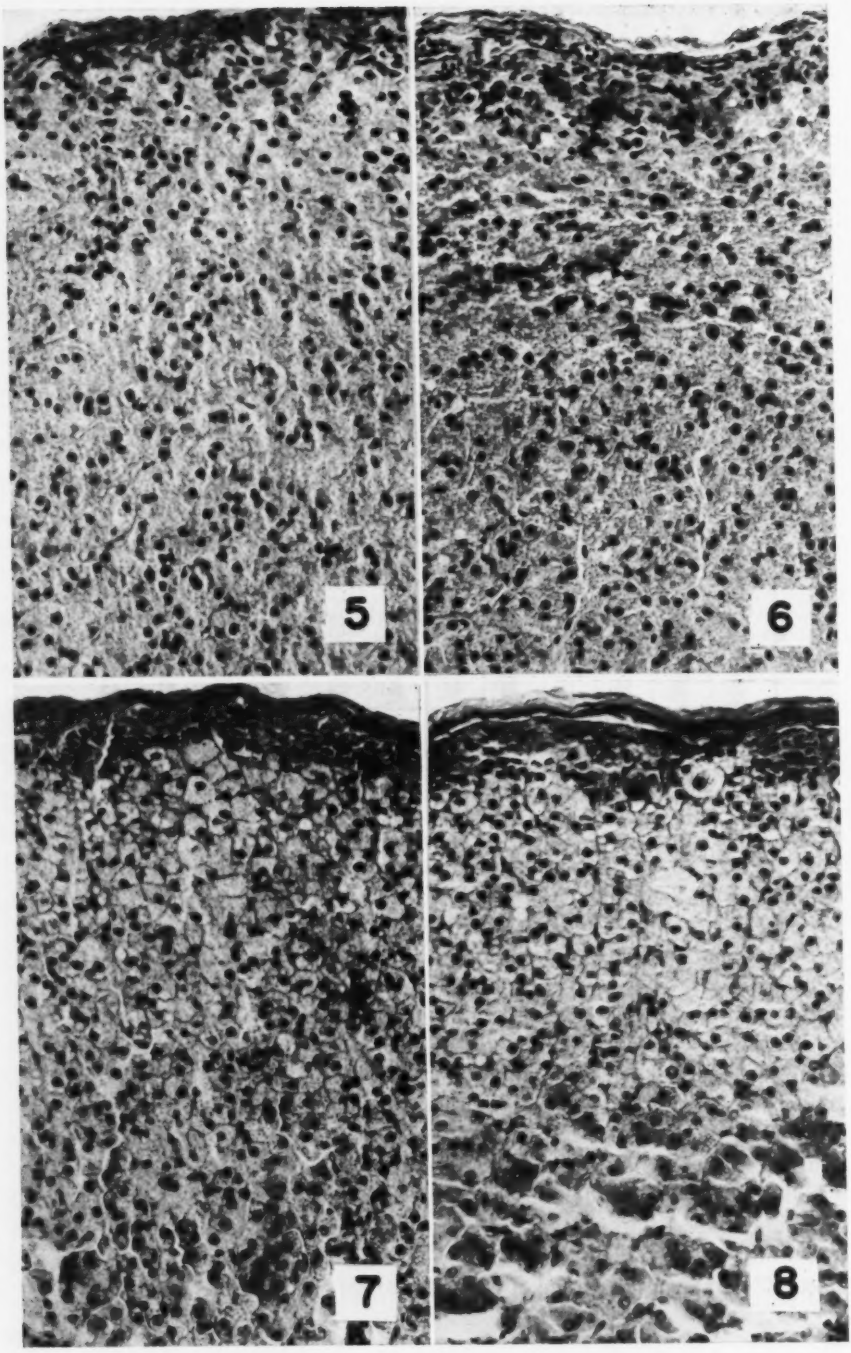


FIG. 4. Ratio of adrenal cholesterol to adrenal ascorbic acid per 100 grams adrenal tissue.

teen days the deficient animals continue to have a high adrenal cholesterol-adrenal ascorbic acid ratio with clinical signs of early scurvy; on the other hand, the partially deficient guinea pigs have a sudden increased ratio but no obvious clinical signs of scurvy. The control animals continue to have a low ratio. At day twenty-one the deficient animals have frank scurvy and a very high adrenal cholesterol-adrenal ascorbic acid ratio. The partially deficient guinea pigs clinically are beginning to show very early signs of scurvy and their ratio appears to be elevated to the 500 mark. This is in keeping with the data of Crampton (1947) that 0.5 mg. ascorbic acid daily intake is a minimal maintenance level and that, as there is growth of the animal, the intake has to be increased above this level.

The morphological changes in the adrenals parallel to a certain extent the biochemical findings. There were no striking differences in all groups of animals after seven and fourteen days on the various ascorbic acid intakes The gross appearance of the adrenals of the deficient animals is that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of slight hypertrophy, while in the other groups the adrenals are comparatively normal. After twenty-one days, striking changes from the normal gross and histological picture are noted. Grossly, the adrenals of deficient animals are pale, enlarged, and contain small hemorrhages. Only a moderate enlargement is seen in the partially deficient guinea pigs. The adrenals of the normal and those of the fortified animals are grossly normal.

Histologically, the adrenals of the deficient animals (Fig. 5) have an essentially normal zona glomerulosa. The zona fasciculata is thickened and the normal radial arrangement of the cells is somewhat disorganized, the cells often presenting a poorly defined cell membrane and small nuclei.

The adrenals of the partially deficient guinea pigs present a similar picture, although disorganization of the cord-like arrangement of the cells is limited to the outer fasciculata (Fig. 6). The normal adrenal cortex (Fig. 7) when compared to that of the fortified animals (Fig. 8) has a thicker zona fasciculata; however, the lipid granules, cell boundaries, nuclei, and architecture are well preserved in the adrenal cortices of both groups.

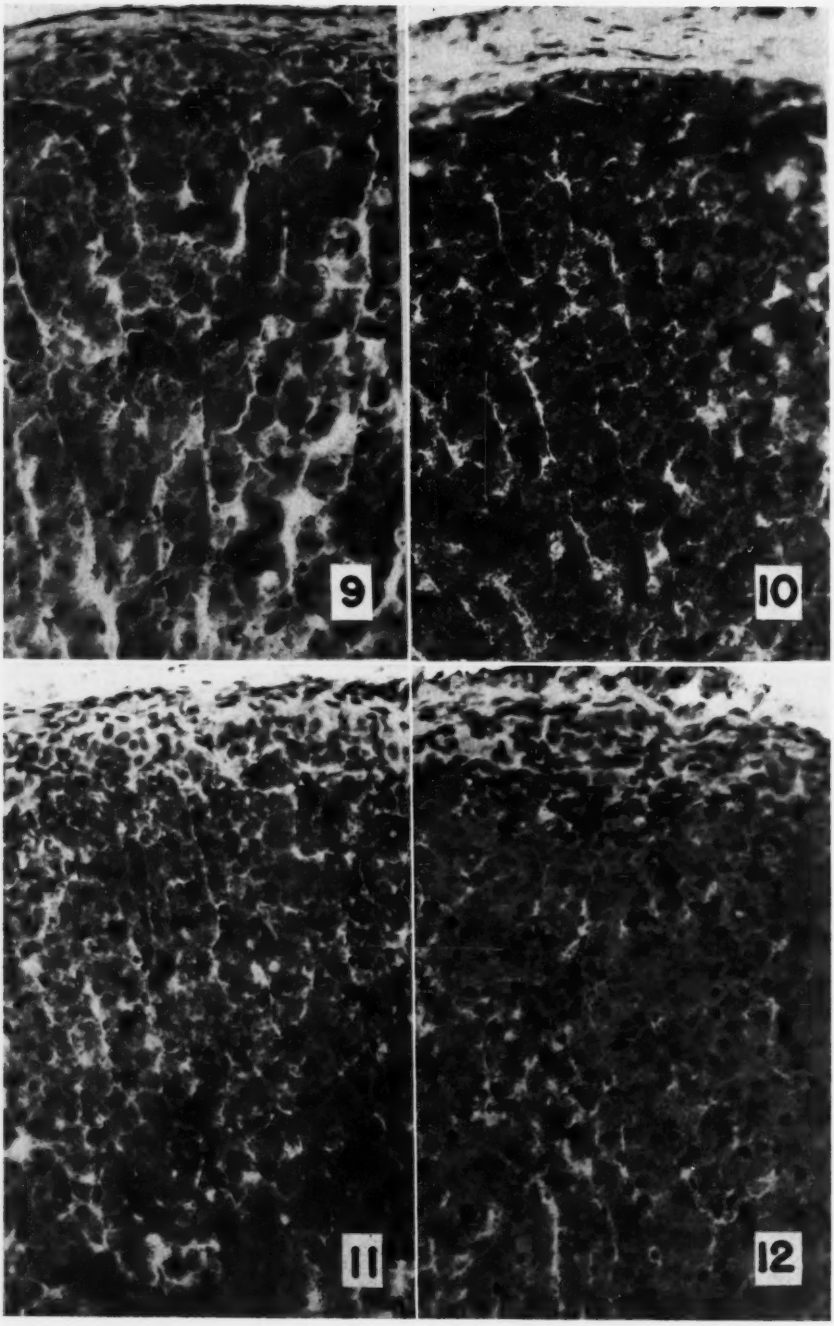
Frozen sections stained with Oil Red 0 demonstrate sudanophilia in all groups. In the deficient animals there is a loss of large fat droplets, and the amount of sudanophilic material is decreased (Fig. 9). A considerable concentration of lipids, however, persists in the outer fasciculata. The sudanophilia in the partially deficient animals' adrenal cortex is diffuse with absence of large fat droplets (Fig. 10). The adrenal cortex of the fortified guinea pigs is somewhat thinner than normal; however, there is prominent sudanophilia throughout, particularly in the inner fasciculata (Fig. 11). The normal adrenal cortex has a wide distribution of sudanophilic material (Fig. 12). From these data it appears that, contrary to the studies of Weaver and Nelson (1943) and of Deane and Morse (1948), the arrangement of adrenocortical lipids in fine droplets is indicative of either a state of activity or of "exhaustion" and, probably, the large droplets of lipids represent storage. This assumption is partially supported by the birefringency patterns.

FIG. 5. Adrenal cortex of guinea pig deficient in ascorbic acid for 21 days. Normal zona glomerulosa. Wide zona fasciculata with disorganized cell pattern. Hematoxylin and Eosin. ×265.

FIG. 6. Adrenal cortex of guinea pig partially deficient in ascorbic acid (0.5 mg./day) for 21 days. Normal zona glomerulosa. Disorganized cell pattern in the outer fasciculata. Inner zone is normal. Hematoxylin and Eosin. ×265.

FIG. 7. Adrenal cortex of guinea pig receiving 2.5 mg. ascorbic acid/day for 21 days. Normal zona glomerulosa and zona fasciculata. Hematoxylin and Eosin. ×265.

FIG. 8. Adrenal cortex of guinea pig receiving 25 mg./day (fortified) ascorbic acid for 21 days. The zona fasciculata is thin with a normal architectural pattern. Zona glomerulosa is normal. Hematoxylin and Eosin. ×265.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The birefringency of the adrenal cortex of the deficient animals is markedly reduced (Fig. 13). In a few scattered areas foci of double refractile bodies can be observed. This is, however, a depleted cortex when compared with the normal birefringence pattern (Fig. 14). A less prominent reduction in the number of birefringent particles is seen in the partially deficient guinea pigs' adrenal cortex (Fig. 15). The greatest concentration of birefringent particles in the fortified animals is in the inner fasciculata, conforming to the pattern of sudanophilia (Fig. 16).

In all adrenal sections, except those of the deficient animals, the Schultz test is positive. The partially deficient animals' adrenal cortices, although not as reactive, are definitely positive. Histochemically demonstrable ascorbic acid was seen only in the fortified adrenal cortices.

In order to determine whether or not the changes in the adrenal cholesterol are organ specific, liver and serum cholesterol analyses were made. The most striking changes are tabulated for day twenty-one (Table 3). The total liver cholesterol of the deficient animals is consistently higher than that of their pair-fed controls. This is observed particularly at day fourteen and to a lesser degree on days seven and twenty-one. It should be noted that there is a greater proportion of free cholesterol both in the totally and in the partially deficient animals beginning at day fourteen. This condition has, however, stabilized itself in the partially deficient guinea pigs at day twenty-one. The serum cholesterol, on the other hand, shows no significant changes except at day twenty-one in the deficient animals and in their pair-fed controls where there is a significant and unexplainable increase.

The liver glycogen of the deficient animals is decreased after seven days of deficiency and reaches a low level at days fourteen and twenty-one (Table 3). This is concomitant with the appearances of clinical scurvy. At day fourteen the liver glycogen of the partially deficient animal is reduced, but is higher than that of the seven-day totally deficient guinea pigs. However, at day twenty-one the liver glycogen is within normal limits. The depletion of the liver glycogen is paralleled by the increase in liver cholesterol. The liver glycogen level of the pair-fed control animals is always within normal limits.

FIG. 9. Adrenal cortex of guinea pig deficient in ascorbic acid for 21 days. There is a loss of large fat droplets. The amount of sudanophilic material is reduced. Oil Red 0. ×285.

FIG. 10. Adrenal cortex of guinea pig partially deficient in ascorbic acid for 21 days. There is absence of large fat droplets. Sudanophilic substance is diffuse. Oil Red 0. ×285.

FIG. 11. Adrenal cortex of guinea pig fortified with ascorbic acid for 21 days. Prominent sudanophilia in the zona fasciculata. Oil Red 0. ×285.

FIG. 12. Adrenal cortex of guinea pig (Normal). Wide distribution of sudanophilic material. Oil Red 0. ×285.

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TABLE 3. SOME EFFECTS ON LIVER AND BLOOD OF VARIOUS LEVELS OF ASCORBIC ACID INTAKE* (21 DAYS)

Ascorbic acid intake, mg./day	Liver wt., gm./100 gm. body weight	Liver cholesterol, mg./100 gm.		Liver glycogen, gm./100 gm.	Serum cholesterol, mg./100 cc.		Blood glucose, mg./100 cc.	Serum sodium, Me/L
		Free	Total		Free	Total		
0.0	4.19±0.32	185±15	336±17	0.44±0.06	17±3.0	61±4.3	88±0.1	136.5±0.2
2.5 pair fed	3.80±0.40	169± 9.5	306±32	2.55±0.08	12±2.4	55±6.0	64± 1.0	134.7±0.4
0.5	4.26±0.60	51±18.3	263±18.6	2.45±0.48	9±1.0	35±4.2	51± 8.6	138.4±2.1
2.5 pair fed	4.02±0.36	23± 7.3	211±23.4	2.39±0.98	6±3.0	25±2.5	64±14.7	136.2±3.1
25.0	4.13±0.10	40± 9.1	209±12.9	2.71±0.60	5±1.5	31±1.5	86±17.2	134.3±2.1
2.5 pair fed	3.74±0.06	82±34.8	229±38.6	3.25±0.59	5±1.5	34±4.3	108±21.5	134.2±2.9

* Six animals in each group.

The liver glycogen concentration has been shown to be dependent in part upon the gluconeogenetic properties of the cortisone-like hormones (glucocorticoids). When there is a diminished output of glucocorticoids there is a decrease in liver glycogen. In the partially deficient animals there is a diminution of the liver glycogen. However, the adrenal gland appears to be responsive to further stimulation by the pituitary (stress?) as shown by the fact that at day twenty-one there is a compensatory increase in liver glycogen apparently due to increased adrenal glucocorticoid output. (Long, Katzin, and Fry 1940). At day twenty-one the liver glygocen is the same as that of the fortified guinea pigs. The serum sodium levels are within the normal range in every group of animals, and for all three periods (Table 3).

The liver is reduced in size in the totally and partially deficient animals but is normal in the fortified guinea pigs. The weight of the spleen tends to increase in the depleted animal. The difference is not statistically significant and is due, in part, to the presence of hemorrhages in the splenic pulp. The controls have normal spleens. The thymus and lymph-nodes show no remarkable changes except perhaps a moderate enlargement of the mesenteric glands in the deficient guinea pigs.

There appears to be no correlation between ascorbic acid intake and the lymphocyte count, taking into consideration both absolute and relative values. Direct eosinophil counts disclose a diminution of the number of these cells in the totally and in the partially deficient animal when compared to that of the normal guinea pigs. The fortified animals show an

FIG. 13. Adrenal cortex of guinea pig deficient in ascorbic acid for 21 days. Birefringency is markedly reduced. Unstained frozen section, polarized light. ×285.

FIG. 14. Adrenal cortex of guinea pig. Normal birefringency pattern. Unstained frozen section. Polarized light. ×285.

FIG. 15. Adrenal cortex of guinea pig partially deficient in ascorbic acid for 21 days. Diminished birefringency. Unstained frozen section. Polarized light. ×285.

FIG. 16. Adrenal cortex of guinea pig with fortified ascorbic acid intake for 21 days. There is concentration of refractile bodies in the inner fasciculata. Unstained frozen section. Polarized light. ×285.

increase at days 7 and 14, followed by a precipitous drop at day 21. The differences between the various groups, however, are not statistically significant. In our opinion, this test cannot be relied upon in the guinea pig because of the extreme variability. This is in accord with the results reported by Brenner *et al.* (Brenner, Waife, and Wohl 1951).

DISCUSSION

From the work of Sayers and coworkers (Sayers, Sayers, Liang and Long 1947; Sayers 1950), there is evidence that the simultaneous depletion of adrenal ascorbic acid and cholesterol without changes of these substances in other tissues is due to pituitary adrenocorticotrophic hormone. In the normal animal this depletion of the adrenal cortex is accompanied by a rise in liver glycogen suggesting that these substances are concerned with the elaboration of the cortical hormones. Further observations by these workers and others (Selye, 1937) have demonstrated that various types of stress, including inanition, cause a depletion of ascorbic acid and cholesterol from the adrenal cortex. In the hypophysectomized animals subjected to similar types of stress there is no depletion of ascorbic acid and cholesterol from the adrenal cortex. However, after the administration of ACTH to these animals there is rapid depletion of the adrenal cortex of ascorbic acid and cholesterol. These facts seem to indicate that the depletion of the adrenal cortex of ascorbic acid and cholesterol is directly associated with increased secretory activity of the adrenal, induced by pituitary adrenocorticotrophin.

Our data show that as the ascorbic acid content of the adrenal cortex is reduced there is a concomitant but less marked reduction in the adrenal cholesterol. When the ratio of adrenal cholesterol to adrenal ascorbic acid has risen from a normal average value of 200:1 to approximately 500:1 a compensatory hypertrophy of the cortex develops. This is accompanied by an almost complete exhaustion of the histochemically demonstrable adrenal cortical steroids and by reduction in liver glycogen. We could not confirm the observation of Long (1947) that the adrenals of scorbutic guinea pigs contain 20 per cent more cholesterol than the pair-fed controls. It is clear, however, that in vitamin C deficiency the depletion of adrenal cholesterol is considerably less and slower than that of ascorbic acid. This contrasts to what happens following ACTH administration, under which circumstances ascorbic acid and cholesterol are decreased approximately to the same degree (Sayers and Sayers 1948; Sayers, Sayers, Liang and Long 1946). Our findings can be interpreted as an indication that in the absence of adequate available ascorbic acid, cholesterol cannot be mobilized for the production of steroid hormones as promptly and completely as in the normal animals subjected to other types of stress. The

report of Long (1947) that administration of ACTH to scorbutic guinea pigs causes adrenal cholesterol depletion and lymphopenia would tend to to invalidate this interpretation. However, Long's report was based on experiments carried out after only 14 days of ascorbic acid depletion when enough vitamin C can still be made available to the organism from tissue stores as evidenced by the absence of manifest clinical and pathological signs of scurvy. Recent work by Oesterling and Long (1951) on acute (17 day) and chronic (28 day) scurvy using ACTH showed that in early scurvy there was no additional depletion of adrenal ascorbic acid after ACTH. However, there was a 42 per cent reduction in the total adrenal cholesterol. In the chronic or late scurvy the levels of adrenal ascorbic acid and adrenal cholesterol appeared to be at a minimal level. In our opinion, the observations of Long do not rule out entirely ascorbic acid as a factor, but would only indicate that extremely small amounts of this vitamin available to the adrenal cortex are adequate for adrenocortical function. Our data, namely, the slowing down of adrenal cholesterol depletion as well as the first appearance of evidence of decreased adrenocortical activity on or about the fourteenth day of ascorbic acid free regimen supports this view. The observations of Daughaday and coworkers (Daughaday, Jaffe and Williams 1948) on the urinary excretion of formaldehydogenic steroids in patients with vitamin C deficiency are difficult to interpret, unless we consider scurvy as an adrenal "stress" as suggested by Nadel and Schneider (1951). The fact that a rise in the elimination of steroids to normal levels does not take place immediately but only after several days of therapy does not exclude a role of ascorbic acid in steroid production as Sayers has interpreted (Sayers 1950). Further analysis of these as well as of our own data would lend support to the hypothesis that the reduction of ascorbic acid in the adrenal cortex of the normal animal is a reflection of ACTH activity but not necessarily indicates synthesis of cortical hormones; the production of corticosteroids, according to this view, would be principally reflected in alterations of adrenal cholesterol content.

The reduction in liver glycogen which was absent in the pair-fed controls can be taken as an indication of a deficient production or activity of glucocorticoids. This interpretation is supported by the fact that histological changes of the adrenal cortex were limited to the zona fasciculata which, according to some investigators (Greep and Deane 1947), is related to the production of 11-oxysteroids. The possibility that the low liver glycogen and the high or normal blood glucose values are due to an effect of ascorbic acid deficiency on insulin production (Banerjee, 1943) should also be considered in evaluating these data. The observations on the interference of phosphorylation of glucose to glycogen due to a decrease

in phosphorylase in the scorbutic animal (Murray, 1950) with hypertrophied islets of Langerhans, however, leads to the postulate that the insulin production may be adequate but the error is in the chain of glycogen synthesis. The exact relationship between vitamin C and carbohydrate metabolism is not clear (Bicknell and Prescott, 1946).

The lack of morphologic changes in the zona glomerulosa as well as the consistently normal sodium values support the view that in ascorbic acid deficiency there is no disturbance of the salt regulating hormones of the adrenal cortex. The paucity of ascorbic acid normally present in the zona glomerulosa (Greep and Deane 1947) supplies additional evidence that ascorbic acid is not essential for the production of the desoxycorticosterone-like hormones which are supposedly produced by the outermost zone of the adrenal cortex.

CONCLUSIONS

It appears that the reduction of steroid concentration in the adrenal cortex during severe ascorbic acid deficiency is due to one or a combination of the two following mechanisms: (1) "exhaustion" of the adrenal cortex due to stress and the continuous production of corticosteroids; (2) diminished production of adrenal corticosteroids in the absence of an adequate concentration of adrenal or otherwise available ascorbic acid. The first mechanism implies that the production of cortical hormones occurs in spite of the gradually decreasing adrenal ascorbic acid concentration. Such a possibility which is supported by recent investigations (Hyman, Ragan, and Turner 1950; Traeger, Gabuzda, Zamcheck, and Davidson 1950; Eisenstein and Shank 1951) seems to indicate that ascorbic acid either is not important in the actual production of the corticosteroids or that other substances may replace the vitamin in hormone synthesis (Sayers 1950). The second mechanism is principally supported by our observations that when the concentration of ascorbic acid and cholesterol reach a certain point there are no demonstrable steroids in the adrenal cortex. The necessity of ascorbic acid being present for the activity of these steroids at the target organs must be mentioned as a possible explanation for the adrenocortical "insufficiency" which is thought to occur in advanced scurvy. This view was supported by earlier observations (McKee, Cobbey, Geiman 1947); however, more recent studies (Schaffenburg, Masson, Corcoran 1950; Pirani, Stepto, Sutherland and Consolazio 1951) seem to disprove this possibility.

SUMMARY

The effect of various levels of ascorbic acid intake on the histology and biochemistry of the adrenal cortex was studied in male guinea pigs. Con-

comitantly various factors such as liver cholesterol and glycogen, serum cholesterol and sodium, blood lymphocytes and eosinophils, and lymphoid tissue, all of which may be affected by adrenocortical activity, were also investigated. An adequate number of pair-fed and fed ad libitum controls were included in this study. Guinea pigs receiving no ascorbic acid had a marked reduction in adrenal ascorbic acid and cholesterol concentrations. After a period of fourteen to twenty-one days this reduction was followed by a rapid fall in liver glycogen. The serum sodium level and lymphoid tissue compartment were not affected. Animals receiving 0.5 mg. daily of ascorbic acid had a decrease in adrenal ascorbic acid but only a slight decrease in adrenal cholesterol and liver glycogen. No decrease was noted in the serum sodium. There was no appreciable change in adrenal ascorbic acid, adrenal cholesterol or liver glycogen in animals receiving 2.5 mg. or 25 mg. daily of ascorbic acid. Histochemical studies on the adrenal disclosed that in the guinea pigs on an ascorbic acid free diet there was a loss of active cortical steroids, as evidenced by the diminution of sudanophilia and the marked reduction of birefringency and Schultz's reacting lipids. Other histochemical methods were used and were not found to contribute any additional information. Similar changes to a lesser degree were seen in the adrenal cortex of the animals receiving 0.5 mg. ascorbic acid per day. From these data it appears that there is an optimal ratio of adrenal cholesterol to adrenal ascorbic acid for normal steroid production in the adrenal cortex. The biochemical and morphological alterations of the adrenal cortex in ascorbic acid deficiency indicate a depletion of demonstrable cortical steroids.

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MECHANISM OF EPINEPHRINE-HYPERGLYCEMIA*

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THE hyperglycemia and glycosuria produced by epinephrine—described half a century ago as "adrenalin diabetes"—has been generally attributed to an increase in the rate of hepatic glycogenolysis. This concept is based on considerable, and apparently unequivocal, experimental evidence. It is well known, for instance, that in hepatectomized animals epinephrine does not produce hyperglycemia, and that in the perfused liver and in liver slices *in vitro* glucose output in increased by epinephrine.

In a recent paper (Somogyi, 1950) we called attention to another, well defined phase of epinephrine action, namely inhibition of glucose assimilation,[1] as a factor that contributes to epinephrine hyperglycemia. In that paper we confirmed the earlier findings of Wilenko (1913), Wiechman (1927), and Cori *et al.* (1928 and 1935), to the effect that epinephrine greatly depresses the rate of peripheral glucose assimilation. We expressed the view that this is a factor which potently enhances hyperglycemia.

The inhibition of peripheral glucose assimilation can be measured by the determination of differences between the glucose concentration in the arterial (capillary) and venous blood (A-V differences). The glycemic level, as is known, is higher in the arterial than in the venous blood. In the post-absorptive state the difference is small, on an average between 4 and 5 mg. per cent; during alimentary hyperglycemia, however, it increases considerably, in rough proportionality to the height of the glycemic level (Somogyi, 1948). This fact is illustrated in the graph, Fig. 1. As the lower pair of curves, I_a and I_v, show, the A-V difference increased to a maximum of 36 mg. per cent, one hour after the oral administration of 100 gm. of glucose, concurrently with a hyperglycemic peak of 130 mg. per cent in the venous and 166 mg. per cent in the arterial blood. In a second test, the same person ingested 50 gm. of glucose and at the same

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[1] To avoid misunderstandings we state that the term, "assimilation," is applied here to that phase of metabolism which is involved solely in the transfer of glucose from extracellular fluids into the cells, a process reflected in the lowering of the glycemic level. We prefer this simple, unequivocal term to the variety of expressions used to denote the same process, such as *removal, withdrawal* of blood sugar, glucose *uptake* or *intake, utilization, metabolization* by the tissues, and even *"burning"* of blood sugar.

time was administered another 100 gm. intravenously, at an even rate, over a 2-hour period. In this manner hyperglycemia was raised to a much higher level than would be possible in a normal person by oral feeding alone. Results of this test are represented by the pair of curves II_a and II_v in Fig. 1. It may be seen that in the second test the hyperglycemic

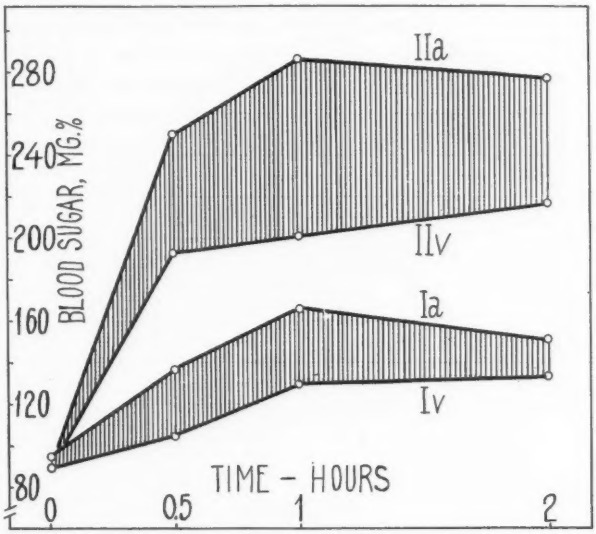

FIG. 1. Two glucose tolerance tests in a non-diabetic young woman. Curves I_a and I_v show changes in arterial (capillary) and venous blood sugar levels after the ingestion of 100 gm. glucose, II_a and II_v after the ingestion of 50 gm. glucose and simultaneous intravenous administration of another 100 gm., infused at an even rate over a 2-hour period. The shaded areas between the arterial and venous tolerance curves ("A-V areas") show the quantitative relationship between the rates of peripheral glucose assimilation in the two tests. Increased hyperglycemia entailed greatly increased rate of assimilation.

peak had been elevated to the abnormal level of 201 mg. per cent in the venous, and 286 mg. per cent in the arterial blood and, concurrently, the maximum A-V difference was augmented to 86 mg. per cent, more than double of the previous maximum. This large A-V difference indicates a great increase in the rate of peripheral glucose assimilation as a consequence of an increase of the hyperglycemic levels. The shaded area, enclosed by the arterial and venous blood sugar curves, the "A-V area," offers a more adequate quantitative picture of peripheral assimilation than single A-V difference values; as may be measured in Fig. 1, this area was increased nearly threefold under the influence of augmented hyperglycemia. The increase in A-V differences, as known, is in the main the

result of insulin action: the higher the hyperglycemic level, the greater is the insulin supply discharged by the pancreas and the higher becomes the rate of glucose assimilation.

An entirely different picture presents itself when epinephrine action, synchronized with glucose feeding, enters as a modifying factor. Under this condition alimentary hyperglycemia is greatly increased, but the A-V difference scarcely deviates from its low fasting level. This fact is illustrated by a representative example given in Fig. 2. The lower pair of curves, I_a and I_v, shows the course of the arterial and venous blood sugar

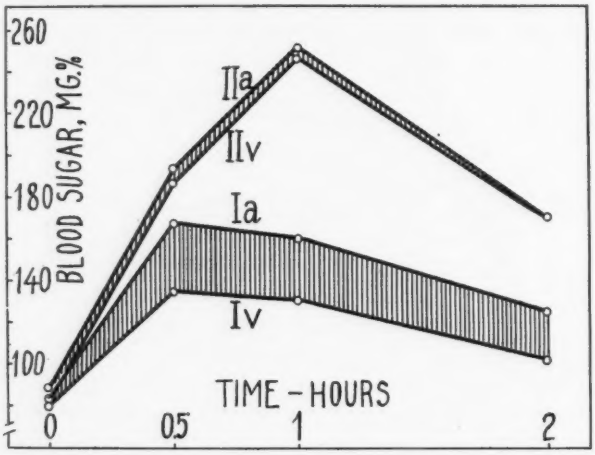

FIG. 2. Two glucose tolerance tests on a healthy man. Curves I_a (arterial blood sugars) and I_v (venous blood sugars) were obtained after oral feeding of 50 gm. glucose, II_a and II_v after the ingestion of glucose was synchronized with subcutaneous injection of 0.2 mg. epinephrine. The A-V area at higher hyperglycemic levels greatly decreased (instead of increasing), as a sign that epinephrine potently inhibited peripheral assimilation.

levels of a healthy young man after the administration of 50 gm. of glucose, while the upper pair, II_a and II_v, represents the results when 0.2 mg. epinephrine was injected a few minutes before the ingestion of glucose. Comparison of Figs. 1 and 2 strikingly illustrates the effect of epinephrine. At normal alimentary hyperglycemia, produced by oral glucose feeding, the rise in both the arterial and venous blood sugar levels, and with it the A-V areas are much of the same order of magnitude in the two graphs. When, however, the arterial hyperglycemia was raised in both experiments to roughly the same maximum level, but in one instance by parenteral glucose administration, while in the other by the injection of epinephrine. the response in peripheral assimilation was vastly different, as may be

clearly seen by comparing the shaded A-V areas (areas between curves II_a and II_v) in the two graphs. Whereas in Fig. 1 the maximum A-V difference was 86, in Fig. 2 it is only 5 mg. per cent, the same as the fasting value. This shows that the action of 0.2 mg. of subcutaneous epinephrine completely counteracted the effect of insulin which—under the stimulus of increased hyperglycemia—was secreted in an increased quantity regardless of the presence of epinephrine.[2] It is evident that while insulin undoubtedly tended to enhance glucose assimilation (via phosphorylation), epinephrine frustrated this process by dephosphorylation, i.e., by liberating glucose from phosphate ester. Thus epinephrine action enhanced alimentary hyperglycemia in the role of an insulin antagonist, by slowing down the assimilation of the glucose that was being absorbed from the intestinal tract.

We are aware that the foregoing experiments do not directly demonstrate that the depression of peripheral assimilation contributes to epinephrine hyperglycemia, i.e., that the phenomenon is not the result of increased hepatic glycogenolysis alone. We undertook, therefore, further studies for clarification of the problem. To this end we determined the hyperglycemic action of epinephrine in the postabsorptive state and during alimentary hyperglycemia, postulating that if increased hepatic glycogenolysis be the sole factor that increases the blood sugar, then hyperglycemia produced jointly by injected epinephrine plus ingested glucose cannot be greater than the sum of hyperglycemia produced by epinephrine in the postabsorptive state, plus the hyperglycemia resulting from glucose feeding alone. This assumption is justified by the known fact that hyperglycemia depresses the flow of sugar from the liver (Soskin *et al.*, 1938), hence there is no reason to believe that the effect of epinephrine on hepatic glycogenolysis could be enhanced by simultaneous alimentary hyperglycemia.

In these experiments we adhered to the principles we have consistently followed in all of our studies concerned with the action of various hormones, in that we endeavored to maintain physiological conditions as close to normal as possible. With this in view we avoided large doses so often employed in experiments, which grossly upset the homeostatic balances of the living organism and, in fact, precipitate distinctly toxic, pathologic conditions.

It was found that subcutaneous injection of 0.2 mg. of epinephrine to

[2] The marked hypoglycemia, which as a rule follows in the wake of epinephrine-hyperglycemia, is also indicative of enhanced insulin secretion during the hyperglycemic phase, regardless of the presence of epinephrine. The effect of the increased insulin supply can and does manifest itself only after its antagonist, the injected epinephrine (or at least most of it), has been inactivated.

healthy men weighing between 65 and 75 kilos satisfied this requirement. According to the observations of Trendelenburg (1923) this dose is on the borderline, representing the minimum which produces detectable effects in adult men. Cori and Buchwald (1930) found, furthermore, that 0.2 mg. epinephrine given subcutaneously exerts about the same effect as 0.00025 mg. per kilo per minute injected subcutaneously. In subsequent studies Cori and his colleagues (1935) found that intravenous injection of double the minimum dose, i.e., 0.005 mg. per kilo per hour, caused an increase in blood pressure of only 10 mm. "In such a case," they stated, "one finds a decrease in the blood flow through the leg, so that the sugar uptake is actually smaller than is indicated by the arteriovenous differences." Observations on our subjects were in line with these older findings: they failed to reveal any changes in heart rate or blood pressure that were even near to being significant. The changes in glucose assimilation, on the other hand, as reflected in hyperglycemic levels and arteriovenous differences in our experimental procedure, were of such magnitude that at least a five-fold increase in the rate of blood flow would be required to produce the narrowing down of the arteriovenous differences to the extent observed in our studies.

We place emphasis, furthermore, on observing hormone action in the intact organism in its normal environment, where, as is known, the action of epinephrine is closely interrelated with that of the hypophysis and other endocrine organs. For this reason we employed in all of our experiments healthy young men (mostly physicians), the subject undergoing three

TABLE 1. COMPARISON OF HYPERGLYCEMIC EFFECT OF EPINEPHRINE, OF GLUCOSE FEEDING, AND OF THE TWO FACTORS COMBINED

Subject No.	Time, hours	Mg. glucose per 100 cc. venous blood			Increase of blood sugar above fasting level, mg. per cent		
		Test 1 Epinephrine alone	Test 2 Glucose alone	Test 3 Epinephrine +glucose	Test 1	Test 2	Test 3
I	0	89	88	94	0	0	0
	0.5	116	131	196	27	43	102
	1	107	116	204	48	28	110
	1.5	87	88	138	−2	0	44
	2	88	—	96	−1	—	2
II	0	92	90	89	0	0	0
	0.5	102	146	188	10	56	109
	1	114	136	242	22	46	153
	1.5	107	112	160	15	22	71
	2	98	98	113	6	8	24
III	0	92	86	96	0	0	0
	0.5	102	127	172	10	41	76
	1	103	125	209	11	39	113
	2	90	90	108	−2	4	12

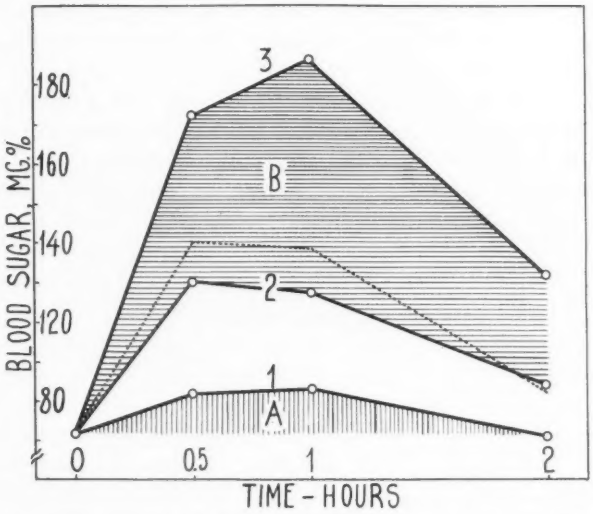

FIG. 3. Glucose-time curves obtained in three tests on a healthy man. Curve 1 resulted after the subcutaneous injection of 0.2 mg.epinephrine, Curve 2 after ingestion of 75 gm. glucose, Curve 3 after glucose feeding was synchronized with the injection of epinephrine. Area *A* (vertically shaded) represents the amount of excess glucose in circulation as a consequence of epinephrine action; it is attributed to increased hepatic glycogenolysis. Area *B* (horizontally shaded) represents the excess glucose in circulation as a result of epinephrine action during alimentary hyperglycemia. The dotted curve was so constructed that the area enclosed by it and by Curve 2 equals area *A*. The third test (glucose feeding and epinephrine injection) would produce the dotted curve instead of Curve 3 if the excess sugar in circulation were produced by hepatic glycogenolysis.

tests, each performed in the morning before breakfast. In the first test 0.2 mg. epinephrine was injected subcutaneously, in the second the subject received 75 gm. of glucose by mouth; finally, in the third test the same amount of glucose was given but was preceded by the injection of epinephrine, i.e, Test 2 was superimposed on Test 1. In all three tests venous blood sugar was determined at 30-minute intervals after the ingestion of glucose.

In Table 1 are recorded the results of three such experiments. A glance at these figures reveals that the hyperglycemic effect of epinephrine was much greater after glucose feeding than in the fasting state. In Subject 1, for instance, one hour after the injection of epinephrine (Test 1) the blood sugar was 18 mg. per cent above the fasting level, and one hour after glucose feeding (Test 2) the increase was 28 mg. per cent. The sum of the two quantities, $18+28=46$ mg. per cent, then, should be the approximate increase at the corresponding time interval in Test 3. Instead, this increase was 110 mg. per cent, more than double what it would be if hepatic

glycogenolysis alone were accountable for the hyperglycemic action of epinephrine. It is evident that a considerable part of the hyperglycemic effect of epinephrine must be attributed to the inhibition of glucose assimilation. This factor contributes to hyperglycemia not by increasing the flow of sugar from tissue cells into the blood (glycogenolysis), but by inhibiting the assimilation of glucose, i.e., by slowing down its transfer from extracellular fluids into tissue cells.

For clearer visualization a typical experiment in this series is presented graphically in Fig. 3. Curve 1 shows the extent of hyperglycemia produced by the injection of 0.2 mg. of epinephrine in the postabsorptive state (Test 1), the vertically shaded area *A* representing in fair proportionality the quantity of excess glucose thrown into circulation by epinephrine action. Curve 2 shows the course of hyperglycemia after glucose feeding (Test 2), and Curve 3 was obtained when ingestion of the same amount of glucose was preceded by the subcutaneous injection of 0.2 mg. of epinephrine (Test 3). The shaded area *B* between Curves 2 and 3 is proportional to the amount of excess glucose in circulation as the result of epinephrine action. If this action would simply consist of an acceleration of hepatic glycogenolysis, which is quantitatively represented by the area *A*, then area *B* would have to be of the same order of magnitude as area *A;* that is to say, the glucose tolerance curve in Test 3 would take the course indicated by the dotted curve in Fig. 3, which was so constructed that the area between it and Curve 2 is identical with area *A*. The fact that area *B* in our several experiments was from 3.5 to 6.7 times greater than area *A*, means that under our experimental conditions hepatic glycogenolysis accounted for only a fraction of the increase in alimentary hyperglycemia under the impact of epinephrine action; the balance was the result of depression of the rate of assimilation.

In a previous paper (Somogyi, 1950) we advanced the view that since "glucose assimilation in the liver is managed essentially by the same enzyme system as in the peripheral tissues, the inference is virtually inescapable that a factor which inhibits glucose assimilation in the peripheral tissues exerts the same influence in the liver." This assumption—which, we feel, is justified by known facts—implies that the postabsorptive epinephrine-hyperglycemia (represented by area *A* in Fig. 3) is not necessarily the product of increased hepatic glycogenolysis alone, but at least in part may be due to an inhibition of hepatic glucose assimilation. We are persuaded that, in general, whenever the rate of flow of sugar from the liver is under investigation, it is not permissible to attribute any observed changes solely and simply to changes in the rate of glycogenolysis, but possible (and highly probable) changes in the rate of hepatic glucose assimilation must also be taken into account. This aspect of the problem,

thus far completely overlooked, invites further experimental studies under physiological conditions.

SUMMARY

Experimental evidence is submitted to show that depression of the rate of peripheral glucose assimilation by epinephrine action plays an important part in the production of epinephrine-hyperglycemia.

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DUAL CONTROL OF ADRENOCORTICOTROPHIN RELEASE*

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THE release of ACTH from pituitary transplants, in response to various stressing agents (Cheng *et al.*, 1949; Fortier and Selye, 1949; McDermott *et al.*, 1950, A, B), was accepted as strong evidence against participation of the hypothalamo-hypophyseal pathways in the stress-induced activation of the pituitary-adrenal system (McDermott *et al.*, 1950, B; Sayers, 1950, 1951). Further observations would seem to warrant a reappraisal of the problem leading to a different interpretation. Exposure of rats to intense sound or light was shown in this laboratory to elicit a rapid and intense discharge of ACTH, as evidenced by the depletion of the adrenal ascorbic acid content (Fortier, 1951, A, B). Similar results were reported by the Cambridge group with the emotional stress of immobilization which induced a marked lymphopenia in the rabbit (Colfer *et al.*, 1950). Administration of cortisone (Fortier *et al.*, 1951, A, B), adrenodemedullation or the use of DIBENAMINE, an adrenolytic agent (Fortier, 1951, A, B), proved, in our hands, equally ineffective in preventing the sound-induced adrenal ascorbic acid depletion, which was, however, suppressed by transplantation of the pituitary from its normal site into the anterior chamber of the eye (Fortier, 1951, A, B). Likewise, as shown by the Cambridge workers, the emotionally elicited lymphopenia of the rabbit, persistent after denervation of the adrenal gland (Colfer *et al.*, 1950), "was abolished by lesions in the pars tuberalis and, in most cases, was abolished or diminished by transverse lesions in the posterior region of the tuber cinereum or in the mammillary body" (De Groot and Harris, 1950). The corticotrophic effect of such neurotropic stimuli as sound and immobilization was apparently dependent on the integrity of these very hypothalamo-hypophyseal pathways whose suppression proved compatible with the release of ACTH in response to systemic stimuli

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(cold, histamine, adrenaline) (Cheng *et al.*, 1949; Fortier and Selye, 1949; Fortier, 1950, B; McDermott *et all*, 1950, A, B; Selye and Fortier, 1950). Hence the following experiment in which the corticotrophic effect of the two varieties of stimuli, as judged by the fall of the circulating blood eosinophils, was compared, after separation of the adenohypophysis from the hypothalamic centers through its transplantation into the anterior chamber of the eye.

METHODS

Preparation of the animals.—Male Piebald rats of an average initial weight of 100–120 g. were used. Adeno-hypophyseal transplants were aseptically introduced, under light ether anesthesia, into the anterior chamber of either one or both eyes, according to a previously described technique (Fortier and Selye, 1949). Animals of the same sex and weight served as donors. Two days later, hypophysectomy was performed by the standard parapharyngeal approach. As a preventive measure, on the day of the operation and at weekly intervals for the following six weeks, penicillin in oil was injected subcutaneously at a dose of 50,000 units. The animals were kept, for the duration of the experiment, at a constant room temperature of 28° to 30° C. They were given a 5% glucose solution to drink and maintained on a diet of dry PABLUM.

Stimuli.—Between 45 to 55 days postoperatively, the tests were initiated on 22 animals whose pituitary transplants showed adequate morphological take, and on eleven intact controls. They were repeated at weekly intervals with different stimuli including adrenaline (epinephrine HCl, 0.02 mg./100 g. dissolved in 0.2 cc. of 0.9% NaCl and injected subcutaneously), histamine acid phosphate (1 mg./100 g. also dissolved in 0.2 cc. of 0.9% NaCl and administered intraperitoneally), audiogenic stimulation (30 minutes of exposure to sound generated by a type 2, 2 H.P., 110 V., 25 Amps., Federal Siren), cold (3 hours of exposure at 0° to 2° C.) and immobilization (3 hours). The latter stress was induced by firmly wrapping up the animal with adhesive tape in a dorsal decubitus and in such a way as to allow for ample breathing while preventing any change in position (Mann and Lemonde, 1951). All of the animals were alternately exposed to the adrenaline, sound and immobilization stimuli. Two sub-groups, each made up of one half of the experimental group, underwent respectively either the cold or the histamine test. The adrenaline test was repeated, in a final stage, on six animals whose graft-containing eye had been enucleated on the previous day.

Eosinophils.—Immediately before and three hours after initiation of the stimulus, direct counts of blood eosinophils were made on freely flowing samples of tail blood by the selective method of Randolph (1949). Uniform diluting pipettes were utilized. The mixing of the blood was standardized by the use of a BURTON pipette-shaker and the eosinophils were counted on both sides of a LEVY-NEUBAUER counting chamber of 0.2 mm. depth. A rather wide range of variation was observed in the initial levels of the eosinophils in both intact (100–275/mm.^{3}) and pituitary-grafted (75–450/mm.^{3}) animals. However, the high correlation coefficient, observed by the YALE workers between the initial levels and the absolute falls of these elements, "makes it statistically permissible to employ percentage fall as an accurate index of change" (McDermott *et al.*, 1950, B). Our results have been so expressed.

Histology.—The animals were killed by bleeding at the end of the experiment. The adrenals and testes were fixed in BOUIN'S fluid prior to weighing. The graft containing eye (or eyes) was removed, its posterior half cut away and the remaining portion fixed

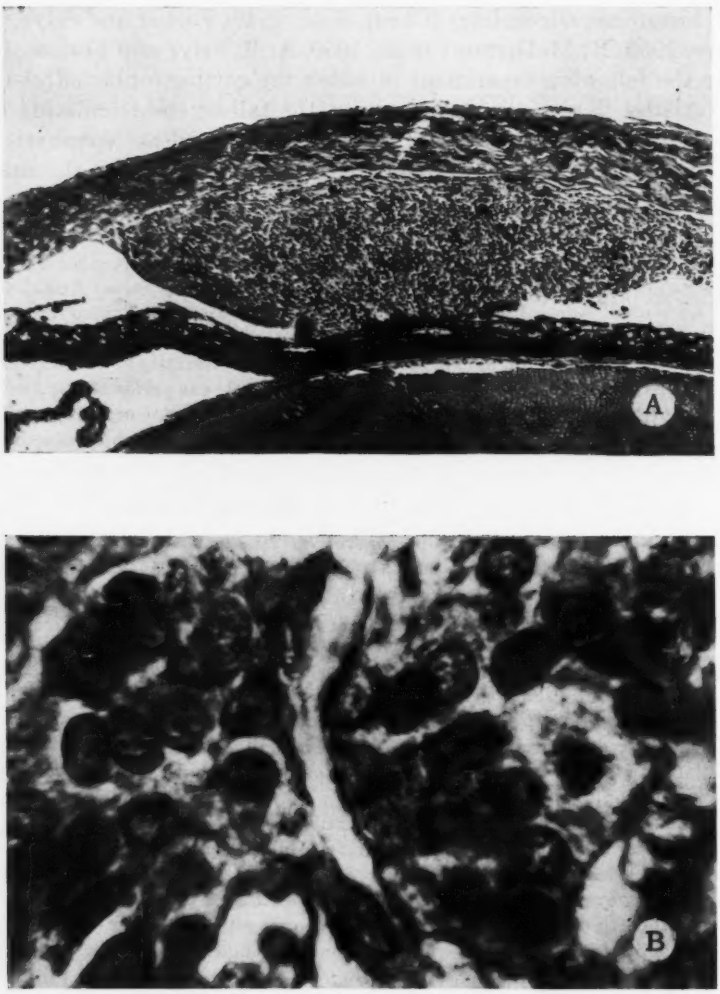

FIG. 1. a) Adenohypophyseal transplant in the anterior chamber of the eye (50×).
b) Same transplant showing chromophobic cells and clusters of basophils (950×).

in BOUIN-Hollande extemporaneously mixed with sublimate (Cl_2Hg, 10%). The pituitaries of the normal controls and the sellar regions of the hypophysectomized animals were similarly fixed, the latter prior to decalcification. This material, sectioned at 7μ was stained with MASSON'S modified trichrome. Completeness of the hypophysectomy, first checked at autopsy with a 5×magnifying glass, was later ascertained by microscopic examination of serial sections of the sellar region. Pituitary remnants were discovered in four cases and the original experimental group was reduced to the 18 animals which are included in this report.

RESULTS

Histology of the Transplants.—The ocular implants (Fig. 1) were richly vascularized in most cases and presented, in some, various degrees of invasion by a dense connective tissue stroma. Islets of viable glandular tissue showing typical nest formations were found however, in all of them. As in previous studies, the trichrome staining showed a great predominance of chromophobic elements interspersed with rare acidophils, fewer basophils and poorly staining sparsely granulated chromophil cells, whose identity could not be ascertained.

TABLE 1. EFFECT OF PITUITARY TRANSPLANTATION ON BODY GROWTH, ADRENAL AND TESTIS WEIGHT

Groups	Increase in weight	Adrenals, mg./100 g., B.W.	Testes, mg./100 g., B.W.
Controls	199 ± 10	18.5 ± 1	1028 ± 46
Bearers of pituitary transplants	5 ± 4	15.4 ± 1.3	318 ± 50

Body and Organ Weights.—As can be seen from Table 1, the transplants had very little if any effect on body growth and evidenced a markedly deficient gonadotrophic activity, as judged by the prevalent testicular atrophy. By contrast, the adrenal glands showed a much smaller degree of atrophy and were, as a whole, adequately maintained.

TABLE 2. EFFECT OF PITUITARY TRANSPLANTATION ON THE RESPONSE OF THE EOSINOPHILS* TO VARIOUS TYPES OF STRESS

Groups	Adrenaline	Cold	Histamine	Sound	Immobilisation	Adrenaline after enucleation
Controls	−45 ± 5	−43 ± 10	−44 ± 9	−44 ± 7	−76 ± 6	
Bearers of pituitary transplants	−66 ± 5	−52 ± 10	−64 ± 8	+20 ± 16	+ 9 ± 5	+13 ± 10

* Expressed as percentage of deviation from initial level.

Eosinophils.—The response afforded by the circulating eosinophils was well defined (Table 2, Fig. 2). Adrenaline, cold and histamine, the so-called systemic stimuli, induced a marked fall of these elements in both intact and grafted animals. Sound and immobilization, the neurotropic agents, determined a comparable eosinopenia in the controls but none in the pituitary-grafted animals. No fall of the eosinophils was elicited by adrenaline following removal of the graft-bearing eye.

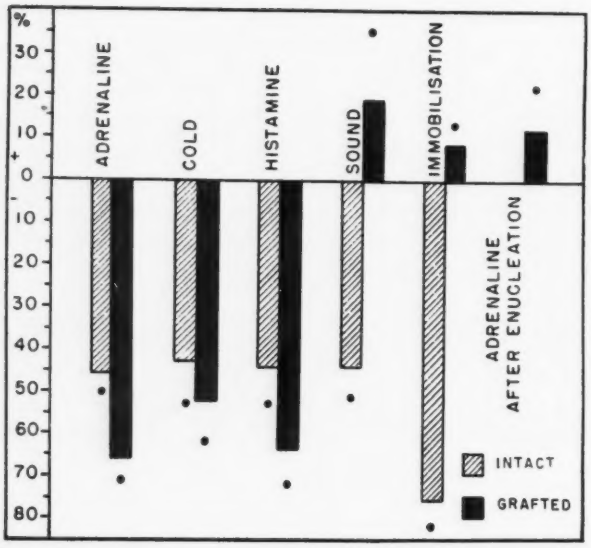

FIG. 2. Comparative response of the eosinophils to neurotropic and to systemic stimuli after hypophyseal transplantation. The percentage of deviation from initial level is indicated by the columns, the standard error by the points.

DISCUSSION

The functional activity of the transplants is of obvious importance in an experiment of this type and forms the prerequisite for its interpretation. From a histophysiological standpoint, the increased CHROMOPHOBIC /CHROMOPHIL cells ration, which has proved in this as well as in previous studies a constant feature of the transplanted pituitary (Cheng *et al.*, 1949; Fortier and Selye, 1949; Fortier, 1950, B; 1951, B), strongly suggests a loss of function which is further borne out by the testicular atrophy and arrested growth of the animals. All the more evident is the repeatedly observed dissociation, in pituitary transplants, between growth and gonadotrophic activities on the one hand, and the corticotrophic function on the other (Cheng *et al.*, 1949; McDermott *et al.*, 1950, A, B; Fortier, 1951, B). The latter was well preserved as judged by the partial maintenance of the adrenals, the stress-induced esoinopenia and its prevention by removal of the graft-containing eye.

Our results, so far in agreement with previous similar studies, point, however, to a definite selectivity in the corticotrophic response to stress of the transplanted pituitary. The lack of response to neurotropic stimuli of otherwise reactive transplants supports our original contention regarding the stimulus specificity of the pathways involved in corticotrophic activa-

tion. At least two different mechanisms of ACTH release are suggested by the above results; the first, induced by so-called systemic agents, would imply the direct activation of the pituitary by one or several humoral agents, the nature of which remains the object of interesting speculations (Gershberg *et al.*, 1950; Long, 1950; McDermott *et al.*, 1950, B; Sayers, 1950, 1951; Vogt, 1950), while the other, brought forth by neurotropic stimuli, would require the participation of the hypothalamo-hypophyseal pathways.

The brilliant investigations of Harris and co-workers, with the stereotaxic stimulation method, point to the posterior region of the TUBER CINEREUM and the MAMMILLARY BODY as the most probable sites for the integration of the nerve impulses (De Groot and Harris, 1950). The latter would be relayed to the adenohypophysis via the hypophyseal portal vessels by means of an unknown neuro-humoral agent (Harris, 1948; De Groot and Harris, 1950). The enhancement of the eserine-induced adrenal ascorbic acid depletion by simultaneous administration of atropine would seem to preclude the possibility of this agent being of a cholinergic nature (Dordoni and Fortier, 1950, 1951). Even though dissociated, under our experimental conditions, the above two mechanisms should not be considered as mutually exclusive in relation to a given stimulus, and it is logical to assume that they are concurrently called into play under the many stress conditions which partake of both nervous and systemic components.

SUMMARY

The ACTH response to systemic (adrenaline, cold, histamine) and to neurotropic (sound, immobilization) stimuli was compared in rats following separation of the adenohypophysis from the hypothalamic centers through homotransplantation of the gland into the anterior chamber of the eye. These animals, along with normal controls, were stimulated at weekly intervals and the fall of the circulating blood esoinophils used as an index of ACTH release. Adrenaline, cold and histamine brought about a definite eosinopenia in both normal and grafted animals. Sound and immobilization induced a marked fall of the eosinophils in the intact but none in the grafted animals. These results suggest a dual regulation of ACTH release, one purely humoral, in response to systemic stimuli, the other, probably neuro-humoral, mediated by the hypothalamo-hypophyseal neurovascular pathways, and coming into play under the influence of nervous and emotional stimuli.

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EFFECT OF ASCORBIC ACID, POTASSIUM CHLORIDE FLOODING, AND CORTISONE ON THE COURSE OF FORMALDEHYDE-IRRITATION ARTHRITIS

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IT HAS been suggested that disturbances in the pituitary-adrenal system may contribute to the adaptation disease of rheumatoid arthritis (Selye, 1950). Whereas cortisone, the glucocorticoid hormone, is known to depress the inflammatory changes, the swelling, and other changes in rheumatoid arthritis, the mineralocorticoid (DCA-like) hormone is known to exacerbate the arthritis. Patients, and rats, given DCA have been reported to develop arthritic lesions (Thorn, 1949; Coutou and Selye, 1950). DCA is also known to increase (Frasinetti and Lanza, 1950), and cortisone to decrease (Selye, 1950), the swelling and other changes in the joint due to topical irritation arthritis. The reported ameliorative effect of combined DCA-ascorbic acid therapy in topical irritation arthritis (Brownlee, 1950) has not been substantiated by subsequent work (Frenk *et al.*, 1950; Frasinetti and Lanza, 1950). Indeed the apparent good effect of the combined therapy in Brownlee's work was probably due to the excess of ascorbic acid instead of to the DCA, since other workers (Dugal, 1951; Frasinetti *et al.*, 1950) observed that ascorbic acid overdosage in rats prevented the swelling due to topical irritation. Furthermore, Frasinetti *et al.* (1950) observed that combined DCA-ascorbic acid therapy prevented the exacerbation of the arthritis otherwise referable to DCA. Littman *et al.* (1951) reported that combined DCA-ascorbic acid therapy was ineffective in patients with rheumatoid arthritis.

The action of ascorbic acid in preventing the swelling of topical irritation arthritis is not known. Several studies have suggested, however, that the vitamin may condition the pituitary-adrenal response to stress; data from this laboratory indicate that pretreatment of rats and mice with the vitamin prevents the adrenal response to epinephrine stress. The vitamin fails to alter the response to adrenocorticotrophin (Bacchus, 1951; Bacchus *et al.*, 1951; Bacchus and Altszuler, 1951). The pituitary-adrenal axis has been shown to be conditioned at a post-adrenal level by potassium chloride flooding (Bacchus, 1951b; Bacchus and Heiffer, 1951). Potassium chloride flooding prevented certain signs of DCA-overdosage in rats. This

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paper reports data on the effects of these conditioning factors, ascorbic acid, and potassium chloride flooding, on the arthritis induced by topical irritation with formaldehyde.

MATERIALS AND METHODS

Female Osborne-Mendel rats weighing about 270 gm. were used in this study. At the beginning of the experiment all animals were injected with 0.1 ml. of 2.5% formaldehyde into the palmar aponeurosis of one leg, the other leg serving as the untreated control. Measurements of the volume of each leg were made just prior to the injection, and once each day subsequent to the injection. The volume of the leg was measured in the following manner. The rat was anesthetized with ether; each leg was immersed up to the knee joint into an overflow cup filled with mercury. The volume of mercury displaced was regarded as the volume of the leg. All rats except those of Group III were on an ad libitum diet of Purina Laboratory Chow and tap water. Group I consisted of 10 rats which received, from the beginning of the experiment, 125 mg. of ascorbic acid (Vitamin C, Injectable, Sodium ascorbate Roche) in daily intraperitoneal injections. Group II (4 rats) were on Chow and tap water and received daily subcutaneous injections of cortisone (Cortone, Merck) at the rate of 3 mg./100 gm. Group III consisted of 6 rats of the same colony as the others but which were flooded with potassium chloride for $2\frac{1}{2}$ months prior to the injection of formaldehyde; potassium chloride flooding (Bacchus, 1950) consisted of an ad libitum fare of Chow and of a 2% aqueous solution of potassium chloride as the sole source of fluid. Group IV consisted of 8 rats which were injected with formaldehyde as above; four of these received daily intraperitoneal injections of saline, and the other four received no injections. These animals as a group served as controls to the animals receiving injectional therapy, and to those flooded with KCl.

Measurements of the legs were made up to 7 days after the injection of formaldehyde; all animals were killed on the eighth day after the injection. The adrenals and parts of the liver were fixed in 10% neutralized formaldehyde. The legs, formaldehyde-injected and the controls, were detached at the knee joint and fixed in Carnoy's fluid. Frozen sections (15 micra) of the adrenals and of the livers were stained with Sudan IV. The periarticular tissue was carefully dissected from the leg and imbedded in Fisher's Tissuemat; 6 micra sections were stained with Delafield's alum hematoxylin and eosin.

RESULTS

The amount of swelling of the legs in the various groups of rats are presented in Table 1. The amount of swelling was calculated from the volume measurements as the difference between the *change* of size of each leg when compared with their sizes on the first day, i.e.,

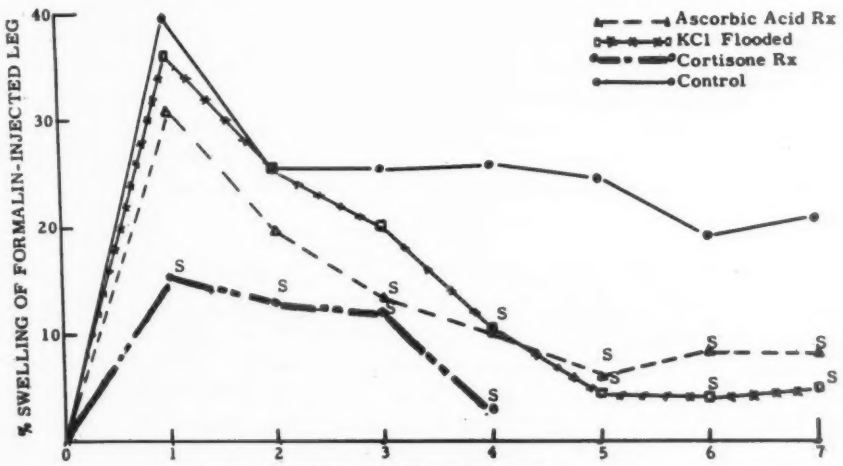
$$\text{Swelling} = (V_{fs} - V_{fo}) - (V_{ns} - V_{no})$$

V_{fo} = volume of formaldehyde-injected leg prior to the injection.
V_{fs} = volume of formaldehyde-injected leg at any instant subsequent to first measurement.
V_{no} = volume of control (normal) leg at first measurement.
V_{ns} = volume of control (normal) leg at any instant subsequent to the first measurement.

TABLE 1. EFFECT OF ASCORBIC ACID, POTASSIUM CHLORIDE FLOODING, AND CORTISONE, ON THE SWELLING DUE TO FORMALDEHYDE-IRRITATION ARTHRITIS

	Swelling of Leg: ml. ±S.E.			
Day	Control (Normal)	Ascorbic Acid	KCl Flooding	Cortisone
1	0.54 ±0.05	0.47 ±0.03	0.51 ±0.07	0.26 ±0.03
2	0.35 ±0.05	0.28 ±0.04	0.34 ±0.06	0.23 ±0.03
3	0.35 ±0.04	0.20 ±0.03	0.30 ±0.06	0.20 ±0.05
4	0.35 ±0.04	0.16 ±0.03	0.15 ±0.02	0.05 ±0.02
5	0.33 ±0.04	0.10 ±0.03	0.07 ±0.03	———
6	0.26 ±0.03	0.11 ±0.03	0.08 ±0.07	———
7	0.28 ±0.04	0.12 ±0.02	0.08 ±0.01	———

By this method of measurement it was possible to eliminate any swelling which could have been unrelated to the injection of formaldehyde. Within one day after the irritation there was a significant increase in the size of the formaldehyde injected legs of Group IV rats; this swelling decreased, but was still significant, by the second day after the injection. Following this, the swelling still significant, reached a plateau. Similar swelling was observed initially in the rats treated with ascorbic acid and in the KCl flooded rats. By the third day after the irritation there was a significant difference ($P < 0.01$) between the degree of swelling in the ascorbic acid treated animals when compared with the controls (Group IV); this significant difference occurred each day until the end of the experiment. The swelling in the legs of the KCl flooded rats was significantly ($P < 0.01$)



Mean percentages are presented ; S = difference from controls is significant.

less than that of the controls from the 3rd day to the end of the experiment. The swelling in the cortisone-treated rat was significantly less than in control animals ($P < 0.01$; on 2nd day, $P < 0.05$). These results are presented as per cent increase in size of the legs in Figure 1.

Gross examination of the legs indicated similar relations. The swelling and glossy appearance of both surfaces of the leg was most severe in the control (Group IV) rats. Signs of hemorrhage of the palmar surface of the leg were present on the first day, and disappeared by the fifth day, of the experiment. The swelling in the rats of Groups II and III was not as severe as in Group IV animals, and signs of hemorrhage disappeared by the second day of the experiment. In three of the four cortisone-treated rats there were no signs of hemorrhage; rather severe hemorrhage occurred in the formaldehyde-injected leg of one animal.

Histological study showed signs of inflammation, increased vascularity, and leukocyte infiltration of the periarticular tissue. The fibrous tissue in these areas seemed to be diminished in amount, the spaces being filled with wandering polymorphonuclear leukocytes, lymphocytes, and histiocytes. These signs also occurred in Groups II and III but were of much lesser intensity. Very little damage was apparent in three of the cortisone-treated rats; one animal showed marked inflammation.

Other Observations. There was increased lipid in the hypertrophic cells of the adrenal glomerular zone of the KCl flooded animals; associated with this was the disappearance of the transitional zone. This appearance confirms a previous finding (Bacchus, 1951b). The zona fasciculata of the adrenals of the cortisone-treated rats showed signs of accumulation of lipid in large globules. The width of this zone was only slightly decreased. There was no marked alteration in the histological appearance of the adrenals of the ascorbic acid-treated animals. There was little change, or a slight increase in the lipid content of the livers of the cortisone-treated animals; the liver lipid content of the other groups was normal.

DISCUSSION

The data on the action of ascorbic acid in this study are at deference to those of other workers who report that ascorbic acid completely prevents the swelling due to topical irritation of the leg. The present study indicates that the swelling is not prevented, but rather, that the recovery is markedly enhanced by ascorbic acid. The other workers did not make daily readings, hence their failure to observe this action of ascorbic acid. The mechanism whereby ascorbic acid diminishes the severity of topical-irritation arthritis is still obscure. Dugal (1951) attempted to explain the action as one of stimulation of the adrenal cortex to produce cortisone-like compounds. It has been demonstrated that ascorbic acid, while altering

the adrenal response to stress, fails to stimulate the adrenal cortex (Bacchus *et al.*, 1951; Bacchus and Altszuler, 1951). The observation that the vitamin probably blocks the pituitary-adrenal axis at a prepituitary level suggests that the vitamin possesses some cortisone-like properties, or some properties synergistic to cortisone. These latter problems are being investigated.

Potassium chloride flooding has been observed to prevent the cardiac hypertrophy, glomerular zone atrophy (Bacchus 1951b) and renal changes (Bacchus and Heiffer, 1951) otherwise referable to DCA overdosage. It is likely that those actions were due to the prevention of a hypokalemia induced by DCA, or to the increased "utilization" of DCA. It is unlikely that the action of KCl observed in this study is due to any direct change in electrolyte balance. It is more plausible that the KCl flooding exerted its action by inducing a persistent mineralocorticoid insufficiency, hence preventing the exacerbation of the arthritis by endogenous mineralocorticoid. The adrenal glomerular zone hypertrophy probably was a compensatory reaction tending to prevent this insufficiency.

SUMMARY

The action of ascorbic acid, potassium chloride flooding, and cortisone on the course of topical formaldehyde-irritation arthritis was studied. Ascorbic acid excess, and KCl flooding, hastened the recovery to normal, but failed to prevent the initial swelling. These treatments also lessened the inflammatory changes in the periarticular tissues of the leg. The swelling and inflammatory changes in the cortisone-treated rats were of lesser intensity than in control animals. The mechanism of the action of ascorbic acid is not known, although data are discussed indicating that it is unlikely that the vitamin stimulates the adrenal cortex. It is suggested that KCl flooding exerts its action by inducing a persistent mineralocorticoid insufficiency.

Acknowledgement

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THE ROLE OF PROGESTERONE IN THE PRODUCTION OF CYCLIC VAGINAL CHANGES IN THE FEMALE GUINEA PIG[1,2]

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THE synergistic action of estrogen and progesterone in the production of growth changes in the uterus and mammary glands of the mouse, rat, rabbit and cat, and in the evoking of heat responses in the mouse, rat, guinea pig and hamster is well known (Hisaw, Meyer and Weichert, 1928; Weichert, 1928; Corner and Allen, 1929; Knaus, 1930; Turner and Schultze, 1931; Turner and Frank, 1932; Turner and de Moss, 1934; Turner and Gomez, 1934; Freud and deJongh, 1935; Hisaw, 1935; Dempsey, Hertz and Young, 1936; Boling and Blandau, 1939; Ring, 1944; Frank and Fraps, 1945). As far as the vagina is concerned, progesterone has been shown to participate in the changes leading to mucification (Hisaw, Meyer and Weichert, 1935; Klein, 1935; Jones and Astwood, 1942).

The possibility that progesterone might have a part in producing the cornification seen during estrus does not seem to have been suggested. The report of Hansel, Asdell and Roberts (1949), based on a study of ovariectomized heifers, appears to be the only one in which progesterone is stated to influence cornification of the vaginal epithelium. When 35 mg. of progesterone were injected daily for 6 days, beginning 2 days after the animal had been brought into heat with diethylstilbestrol, there was an increase in the number of cornified cells which reached a peak late on the second day progesterone was injected. At another time, however, when only diethylstilbestrol was given, an increase in the number of cornified cells was observed about 4 days after the animal was in heat, but to a lesser degree.

The failure of investigators (Coward and Burns, 1927; Kahnt and Doisy, 1928; D'Amour and Gustavson, 1930) to produce complete cornification consistently in 100% of the animals with a given dose of estrogen

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[2] Submitted by the senior author in partial fulfillment of the requirements for the Ph.D. in Anatomy at the University of Kansas, Lawrence, Kansas.

suggests that something else may be involved. A further indication that some other factor besides an estrogen is concerned in producing cyclic vaginal changes was given by our inability to induce normal cornification regularly in ovariectomized guinea pigs injected with α-estradiol benzoate. This inability to produce cornification with predictable regularity by the use of an estrogen was reminiscent of our experience when the induction of estrous behavior was being attempted with estrogen alone. The success which was achieved when the estrogen injection was followed by progesterone (Dempsey, Hertz and Young, 1936; Young, 1941) suggested that the latter hormone might be the factor needed to promote a normally developing cornification.

Observations on pregnant guinea pigs provided an additional indication that progesterone might be this factor. It was previously assumed that the vaginal orifice of the guinea pig remained closed during pregnancy. Recently, however, it has been found that vaginal openings occur in about 80% of pregnant animals. These openings develop at the beginning of the second trimester, when the transition is being made from the ovarian to the placental control of pregnancy (Ford, Webster and Young, 1951). Under the assumption that this was due to a deficiency of progesterone, pregnant animals were injected with this hormone. A decrease in the extent and duration of these openings followed, suggesting, therefore, that the vaginal epithelium is responsive to progesterone as well as to estrogen.

MATERIALS AND METHODS

Ten intact female guinea pigs were used for determining the course of vaginal changes in normal estrous animals. Smears of the vaginal contents were made by aspiration in saline with a glass pipette when the animals were first found in heat and every three hours thereafter for 24 hours. The smears from these animals were air dried on a warming table, stained with hematoxylin and eosin and permanently mounted. This method was used because it was simpler than the more refined procedures of Shorr (1940, 1941) and Papanicolaou (1942) and yet still accurate enough to enable us to follow the cornifying changes during estrus.

As a means of evaluating the smears for the construction of curves that would show the development of the cyclic changes in intact and experimental animals, a scoring method was established based on the Young (1937) modification of the Stockard and Papanicolaou (1917) classification of smear stages. The various stages were rated numerically. One represents the proestrous condition, 2 early cornification with less than 25% of the cells cornified, 3 cornification of between 25% and 75% of the cells, 4 complete cornification, 5 the appearance of pavement cells, and 6 the metestrous leucocytic invasion. In cases where one field of a smear might be scored as 3 and another area as 2 or 4, the mean value of the two fields was used; so that the score might be 2.5 or 3.5. In constructing the curves, the average scores of the vaginal smears from all the animals within a group were plotted against time. As the method worked out, the average score on the ninety-fifth hour, for example, might be 4 indicating full cornification, but not all the animals would have reached this stage. Some would have progressed to the pavement cell stage or to that seen during the metestrum.

The experimental animals consisted of 60 adult guinea pigs ovariectomized when they were about 4 months old. Eighteen or 19 animals were used for each series of injections, and 4 weeks were allowed to elapse before the animals were treated again. The estrogen used was α-estradiol benzoate. It was diluted in corn oil and injected subcutaneously in 3 doses, 24 hours apart. The amounts given were varied, and each injection was usually larger than the one preceding it (Table 1).

TABLE 1. AMOUNT AND TIME OF ALPHA-ESTRADIOL BENZOATE INJECTIONS

Number females	Amount of injected α-estradiol benzoate in I.U. and the time at which it was given		
	0 hr.	24th hr.	48th hr.
37	25	50	100
18	50	50	100
19	50	100	150
18	50	100	200
37	50	150	200
19	100	150	200
18	100	200	200
19	100	200	300
18	100	200	400

Seventy-one hours after the first injection of estrogen, half of the animals received 0.2 I.U. of progesterone[3] in corn oil subcutaneously. The other animals received no additional treatment. Vaginal smears were made immediately prior to the injection of progesterone, and every 3 hours thereafter until the ninety-fifth hour, except the ninety-second hour. The first smears were made on the seventy-first hour because it was noted that rupture of the vaginal membrane occurred in most animals about 70 hours after the initial injection, except when the doses were extremely large (500, 600 and 700 I.U.). Frequently the diestrous condition was seen, but the vaginal epithelium was usually proestrous or in a stage of early cornification with leucocytes still present. The last smears were made at the ninety-fifth hour, because the estrous vaginal changes in intact animals usually are completed within 24 hours following the appearance of the heat responses (Young, 1937).

RESULTS

The changes in the vaginal smears of animals which received only estradiol benzoate are shown in Figure 1. They are to be compared with the changes observed in the intact animals, in which there was a fairly constant progression to the metestrous smear. In most intact animals, the metestrous condition was attained by the seventeenth hour following the detection of heat. The curve then flattened out, showing little change in the next 7 hours.

In no group of spayed animals which received estrogen alone were the changes similar to those in normal animals. A dose of 175 I.U. of estradiol had variable effects. Cornification was seen in only 22% of the females which received this dose, and a more advanced phase was not seen in any of these animals. It is to be noted, parenthetically, that this dose of estro-

[3] Progesterone was supplied by the Schering Corporation, Bloomfield, N. J.

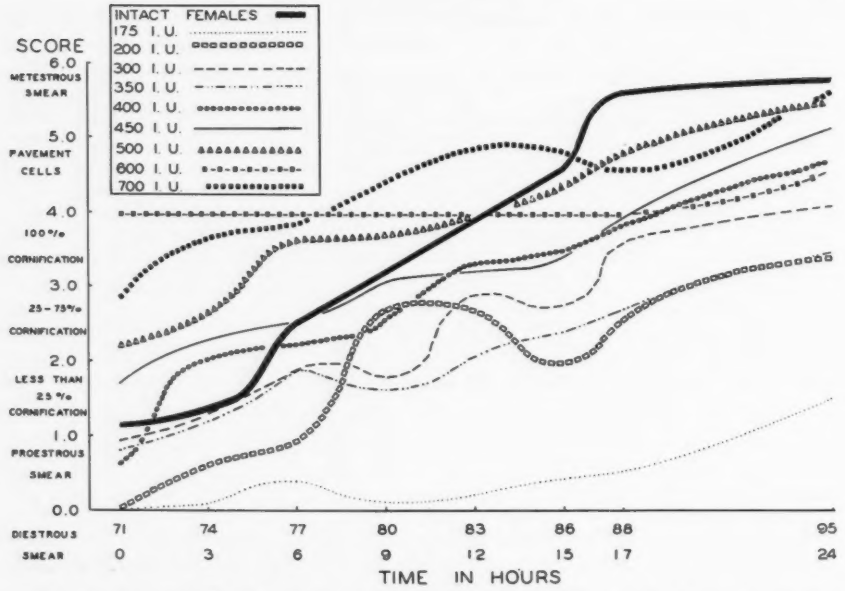

FIG. 1. Response of the vaginal epithelium in spayed guinea pigs given α-estradiol benzoate compared with that in intact estrous females. The time on the abscissa for the experimental animals starts at the seventy-first hour after the first estrogen-injection. Time for the intact animals starts at the zero hour when the animals were first observed in heat.

gen is approximately 11 to 13 times that which will produce full cornification in 50% of spayed rats (D'Amour and Gustavson, 1936) and 2 to 4 times the amount that will condition a female guinea pig for estrus (Dempsey, Hertz and Young, 1936).

When 200 I.U. of estradiol were given, cornification was seen in 44% of the animals. When 300 I.U. were given cornification occurred in 80% of the animals and the metestrous condition in 20%. Following the injection of 350 I.U. of estrogen, only 44% of the animals reached cornification, and none progressed to the stage seen in the metestrum. Four hundred I.U. effected cornification in all the animals treated and the metestrous condition in 33%. Four hundred and fifty, 500 and 700 I.U. brought 66%, 77% and 88% of the animals respectively to the metestrum, but only 44% of the animals receiving 600 I.U. showed the metestrous condition.

Inspection of the data reveal differences between the intact estrous females and the animals given α-estradiol benzoate alone. The vaginal reactions in the latter group were variable and irregular. The variability is revealed by the results obtained from the animals given 350 and 600 I.U.

Irregularities were seen in all groups. The smears of some animals showed an early cornification and remained in this condition throughout the remainder of the experiment. Others never became fully cornified, while others cornified for a short time, regressed, and then became cornified again. The progression of the smear phases in some animals was quite rapid at first and then slowed down or stopped altogether. The reverse was true in others.

Another difference between the smears from the animals given estrogen alone and those from intact females involved the leucocytes. A disappearance of leucocytes is associated with full cornification in 90% of intact females, but was rarely seen in the spayed guinea pigs injected with α-estradiol benzoate. In animals of this latter type cornification of epithelial cells occurred but frequently many leucocytes were present.

The administration of progesterone 24 hours after the last estrogen injection brought about changes in the progression of the smear phases (Fig-

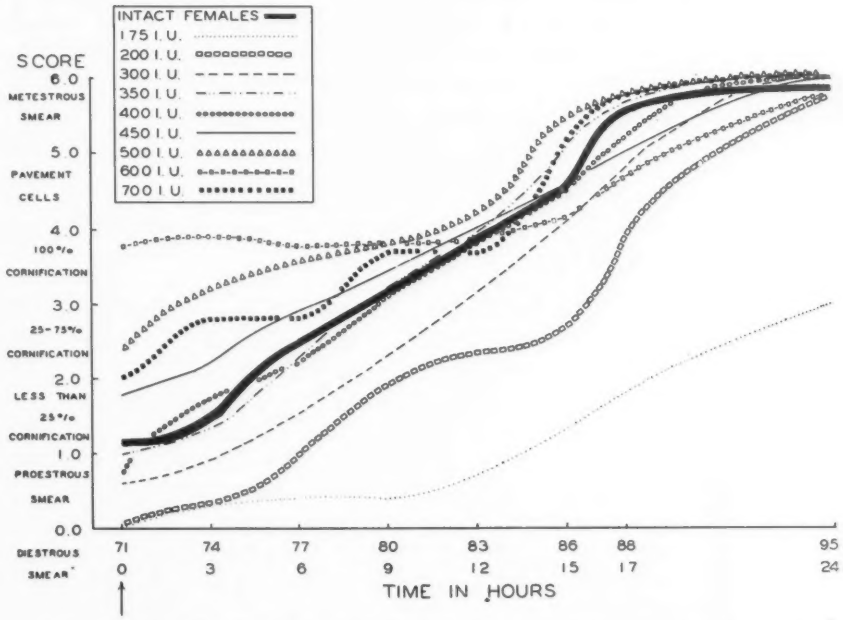

FIG. 2. Response of the vaginal epithelium in spayed guinea pigs given α-estradiol benzoate followed by progesterone compared with that in intact estrous females. The arrow indicates the time progesterone was administered to the experimental animals. The time on the abscissa for the experimental animals starts at the seventy-first hour after the first estrogen-injection. Time for the intact animals starts at the zero hour when the animals were first observed in heat.

ure 2). Most animals, except those which received only 175 I.U. of estrogen prior to the progesterone, went through a stage of complete cornification, pavement cells and the reappearance of leucocytes, in the order seen in intact animals, usually before the ninety-fifth hour. Provided progesterone was preceded by as much as 300 I.U. of estrogen, disappearance of leucocytes at the time of full cornification was seen in 90% of the animals. This compares favorably with intact animals in which leucocyte withdrawal occurred in 90% of the animals. The acceleration effected by progesterone was so rapid that the stage of full cornification was occasionally missed. One smear would show 50 to 60% cornification, and the next smear, 3 hours later, would be in the pavement cell or metestrous stage.

The optimal conditioning quantity of estrogen appeared to be 350 to 400 I.U. When the progesterone was preceded by more than 450 I.U. of estrogen, irregularities associated with the action of estrogen were seen during the first half of the observation period. However, between the ninth and twelfth hour after the injection of progesterone, the smears tended to resemble those seen in intact animals.

As has been indicated, progesterone is concerned not only with cornification, but also with the withdrawal of leucocytes from the vaginal mucosa just prior to full cornification (Figure 3). The solid bar represents the per-

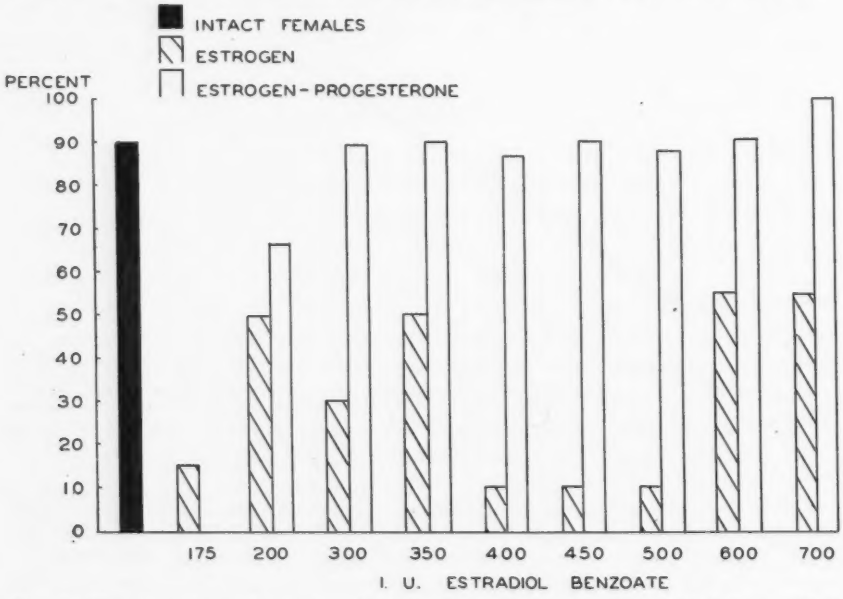

FIG. 3. The percentage of experimentally treated spayed guinea pigs with fully cornified vaginal smears and the disappearance of leucocytes.

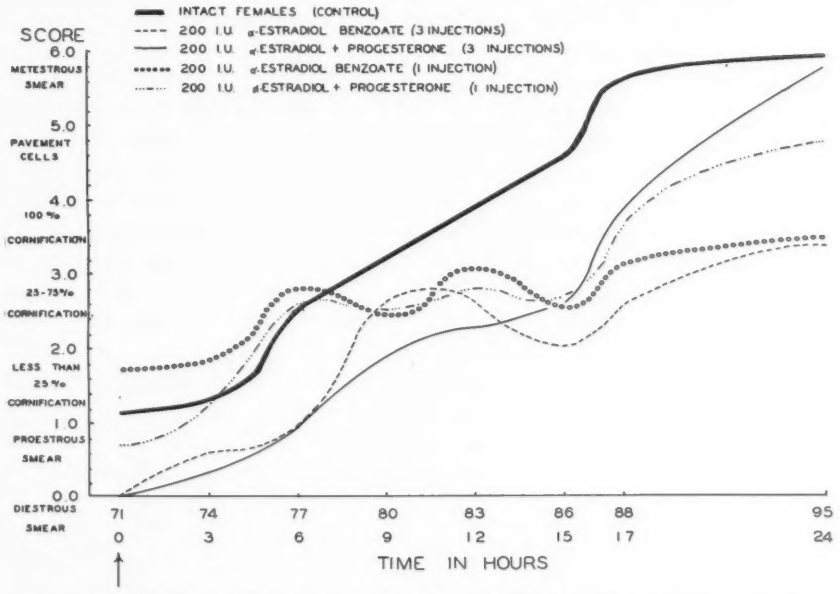
INTACT FEMALES (CONTROL)
200 I.U. α-ESTRADIOL BENZOATE (3 INJECTIONS)
200 I.U. α-ESTRADIOL + PROGESTERONE (3 INJECTIONS)
200 I.U. α-ESTRADIOL BENZOATE (1 INJECTION)
200 I.U. α-ESTRADIOL + PROGESTERONE (1 INJECTION)
SCORE
6.0 METESTROUS SMEAR
5.0 PAVEMENT CELLS
4.0 100 % CORNIFICATION
3.0 25-75% CORNIFICATION
2.0 LESS THAN 25% CORNIFICATION
1.0 PROESTROUS SMEAR
0.0 DIESTROUS SMEAR
71 74 77 80 83 86 88 95
0 3 6 9 12 15 17 24
TIME IN HOURS

FIG. 4. Effect of a single priming dose of α-estradiol benzoate on the vaginal epithelium of spayed guinea pigs compared with the effect of giving the same amount in 3 injections. The time on the abscissa for the experimental animals starts with the seventy-first hour after the first estrogen-injection. Time for the intact animals starts at the zero hour when the animals were first observed in heat. The arrow indicates the time progesterone was administered to the experimental animals.

centage of intact estrous females in which the stage of fully cornified smears without leucocytes was reached. In the case of spayed females which received less than 500 I.U. of estrogen, leucocyte withdrawal never occurred in more than 50% of the animals showing full cornification. When 600 or 700 I.U. of estrogen were injected, the smears from 55% of the animals showing full cornification were free from leucocytes.

When progesterone was administered 24 hours after the last injection of estrogen, leucocyte withdrawal was observed in a consistently high percentage of the animals. The single failure following progesterone treatment was in the group of animals receiving 175 units of estrogen. In none of these animals did the leucocytes disappear. It is apparent that 175 I.U. of α-estradiol benzoate were not sufficient to condition the animals for this action of progesterone.

The effect of a single dose of 200 I.U. of α-estradiol benzoate on the vaginal epithelium is compared with the effect following the same amount of estrogen given in 3 injections (Figure 4). Half of the animals in each group

received 0.2 I.U. of progesterone 71 hours after the first injection of estrogen. In both instances, when the animals were given only estrogen, the irregular type of curve described above resulted. When the 200 I.U. were given in one injection, much of the effect on the vaginal mucosa had already taken place by the seventy-first hour. However, when the same amount of estrogen was given in 3 smaller doses, the initial changes occurred more slowly, and, at the seventy-first hour, the curves lay below those for the animals given the single injection. In both groups, progress beyond the stage reached by estrogen alone depended on the presence of progesterone. It appears furthermore that the administration of 3 smaller doses of estrogen sensitizes the tissues more adequately than the single large dose for the action of progesterone.

Throughout the work, certain structural differences were noted between the cells from animals which received estrogen and those from animals which were injected with estrogen and progesterone. Abnormally shaped and odd-staining cells were present in the smears from the animals treated with estrogen alone. However, as the fixing technique used caused distortion of the cells, it is difficult to determine whether these differences were artifacts of the method or if they were the expression of real structural differences. A further study of the point by more refined methods would be desirable.

DISCUSSION

The data presented indicate that progesterone plays a part in the development of estrous changes in the vagina of the guinea pig. The demonstration of this action brings the vagina into line with the rest of the female genital tract in being reactive to progesterone following its priming or conditioning with estrogen.

Whether the same relationship exists in other species remains to be seen. Suggestive of the possibility for the rat is the difficulty with which cornification is produced consistently with estrogen (Coward and Burns, 1927) and the demonstration that progesterone synergizes with estrogen in the uterus (Hisaw, Meyer and Weichert, 1928; Weichert, 1928), mammary glands (Turner and Schultze, 1931; Freud and deJongh, 1935) and in the tissues mediating estrous behavior (Boling and Blandau, 1939).

Having shown that the vaginal tissue of the guinea pig is sensitive to progesterone, the question arises as to how one would define a guinea pig unit of estrogen. From our data a guinea pig unit could be the amount of estrogen, given in a series of 3 injections of increasing magnitude, followed by 0.2 I.U. of progesterone 24 hours after the last estrogen injection which will effect changes in the vaginal epithelium similar to those observed in the intact animal. These changes are full cornification of the vaginal epi-

thelium with the complete withdrawal of leucocytes, the appearance of pavement cells and the leucocyte invasion characteristic of the metestrum. This series of events was found to be completed in 90% of the females approximately 17 hours after the injection of progesterone when it was preceded by 300 to 400 I.U. of α-estradiol benzoate.

Although the definition of the guinea pig unit is probably of little practical value, it is of interest because of the basis which it establishes for a comparison of the rat with the guinea pig. If the unit for each species is to be based on the experience obtained with estrogen alone, the two species must be contrasted rather than compared, because the guinea pig is so refractory to estrogen. On the other hand, if progesterone acts with estrogen to induce vaginal changes in the rat, a comparison of the two species must await a determination of the hormonal requirements for vaginal changes in the spayed rat that are similar to those seen in the intact estrous female.

SUMMARY

Vaginal changes commonly associated with estrus are not normal when spayed female guinea pigs are injected with 175 to 700 I.U. of α-estradiol benzoate alone. However, when 0.2 I.U. of progesterone is administered 24 hours after the last of 3 estrogen injections given 24 hours apart, the development of the smear phases is rapid and more regular, abnormal cells are uncommon, cornification is complete, leucocytes tend to disappear and there is a progression to the metestrous smear within an interval of time comparable with that in the intact estrous female. The amount of estrogen needed to induce characteristic vaginal changes is greatly reduced. Optimal results were obtained when progesterone was preceded by 350 or 400 I.U. of α-estradiol benzoate.

Treatment of spayed guinea pigs with 200 units of estrogen, given in 3 injections, appears to have sensitized the vaginal mucosa more adequately to progesterone stimulus than did the administration of the same amount of estrogen in one dose.

The vaginal epithelium of the guinea pig is less responsive to estrogen followed by progesterone than are the tissues mediating estrous behavior, 2 to 4 times as much estrogen being required for the normal sequence of vaginal changes.

The demonstration of this synergism between estrogen and progesterone in the production of the cyclic changes in the vaginal epithelium during estrus brings the vagina of the guinea pig into line with the rest of the genital tract in being reactive to progesterone following its priming or conditioning with estrogen.

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THE FIBRINOLYSIN-ANTIFIBRINOLYSIN SYSTEM IN SERUM: MECHANISM OF ITS ENDOCRINE CONTROL[1]

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EARLIER studies have indicated that fibrinolysin (FL), a proteolytic enzyme, appears in blood as a result of the activation of its inactive precursor, profibrinolysin (PFL), by certain stimuli such as the antigen-antibody reaction (Ungar and Mist, 1949). Evidence was presented that the rate of inactivation of fibrinolysin by its inhibitor, antifibrinolysin (AFL), is accelerated by hormones from the pituitary gland and the adrenal cortex. This action appears to be mediated by the spleen since it is absent in splenectomized animals and can be reproduced by a substance present in the spleen, splenin A (Ungar, 1945). In contrast to this accelerating effect of splenin A, a retarding influence was shown by another substance found in the spleen and bone marrow, splenin B (Ungar and Damgaard, 1951).

The present paper deals mainly with endocrine factors which retard the fibrinolysin-antifibrinolysin (FL-AFL) reaction. In view of the close correlation between the rate of the FL-AFL reaction and the intensity of certain inflammatory responses (Ungar, Damgaard and Weinstein, 1951) the observations reported below may suggest a mechanism by which some tissue reactions are controlled by the endocrine system.

METHODS AND MATERIALS

Antifibrinolytic activity can be expressed in terms of the number of units of fibrinolysin inhibited by a given amount of serum: $AFL = FL_o - FL_s$, where AFL is antifibrinolytic activity; FL_o the number of units of fibrinolysin; FL_s the number of units of fibrinolysin left after contact with the serum. It has been emphasized in a previous publication (Ungar and Damgaard, 1951) that the time of contact between FL and the AFL-containing serum is of critical importance in the estimation of the antifibrinolytic activity. When the time of contact is long, for example 20 to 30 minutes, the FL-AFL reaction is nearly completed and the results do not supply any measurement of the rate of the reaction. When, however, the time of contact is reduced to 5 minutes, as in the present work,

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the resulting determinations give measurements of the readily available AFL. As previously reported, a large amount of this readily available AFL indicates a rapid rate of neutralization of FL. The terms, high and low AFL activity, as well as high and low levels of serum AFL, will be used throughout this paper to indicate a rapid or slow rate of the FL-AFL reaction.

In previous studies a viscosimetric method was used for FL estimations. Although otherwise satisfactory, this technique was too time-consuming to be practicable in an extensive study. In its place, a procedure similar to the technique of trypsin estimation described by Kunitz (1948) was adopted. In this procedure, used throughout the present studies, FL acts on fibrinogen; after a given interval the remaining fibrinogen is clotted by thrombin; the incoagulable residue is then filtered. A quantitative determination of the activity of FL is made by measurement of the ultraviolet absorption in the filtrate.

The following reagents are required: (a) Fibrinogen[2] 0.2% in buffered saline (pH 7.4 phosphate buffer diluted with 0.85% Na Cl to 0.15 M); (b) Fibrinolysin[3] 0.1% dissolved in the same buffered saline; (c) Thrombin[4] 2.5% dissolved in acetate buffer at pH 5.6.

To estimate the activity of FL, varying amounts of the solution of FL are pipetted into test tubes and the volumes made up to 3.6 ml with buffered saline. The tubes are then placed in a shaking constant temperature bath (37.5° C.), and 2.0 ml. of the fibrinogen solution is added to each tube. After an interval of 20 minutes each tube receives 0.4 ml. of thrombin and is left in the water bath for another 30 minutes to allow complete clotting of the remaining fibrinogen. The content of each of the tubes is then filtered[5], the filtrate transferred into a silica cuvette and the optical density read at 280 mμ. on the Beckman spectrophotometer. Each sample is read against a blank which contains all the reagent except fibrinogen at the same concentration as the test mixture. The optical densities increase linearly with increasing amounts of FL.

Serum AFL is estimated by the same procedure: samples of the serum to be examined are pipetted into test tubes; each serum is usually tested at three different concentrations (for example 5, 10, and 15 μl., or 0.05, 0.1, and 0.15 ml. of a 1:10 dilution). The tubes are then placed in a water-bath at 37.5° C. and each receives a given amount of FL of known activity. After a contact of *exactly 5 minutes*, 2.0 ml. of fibrinogen is added to each tube and the method proceeds as described in the previous paragraph.

The time intervals must be measured accurately with a stop watch. Timing is begun when FL is added to the first tube and each tube of a series is delayed by 15 seconds. Eighteen samples, that is six sera tested at three concentrations, can be done conveniently in one series. The blank used for AFL estimation also contains the serum.

As stated above, the method is based on the fibrinogenolytic action of FL. It is well known, however, that fibrinolysin also attacks fibrin. It is likely therefore that the enzymatic process does not stop entirely when thrombin is added to the mixture and FL will continue to act on the fibrin clot during the last 30 minutes of incubation. Since this interval is constant and identical for each sample, this additional fibrinolysis does not alter the validity of the results.

Following Kunitz's definition of an enzyme activity unit, the unit of FL was taken as the activity which gives rise under the conditions described to an increase of 1 unit of optical density at 280 mμ per minute of digestion (Kunitz, 1948). AFL activity is then

[2] Bovine Fibrinogen Armour.

[3] Bovine preparation kindly supplied by Dr. E. C. Loomis of the Research Laboratories, Parke, Davis and Company.

[4] Hemostatic globulin kindly supplied by Dr. A. D. Piersma, Lederle Laboratories.

[5] Filter paper S and S No. 597.

defined in terms of the units of FL inhibited by a given amount of serum. This can be calculated by the following formula

$$[\text{AFL}] = \frac{(\text{O.D.})_o - (\text{O.D.})_s}{20}$$

where AFL is the antifibrinolytic activity of a given amount (s) of serum; $(\text{O.D.})_o$ the optical density obtained after action of FL alone (this is kept approximately at 0.45), and $(\text{O.D.})_s$ the optical density reading when the same amount of FL is incubated with serum for 5 minutes prior to digestion.

As noted above, each serum sample is tested at three different concentrations. Since AFL activity is linearly related to the log of the amount of serum used (Ungar and Damgaard, 1951), the three values obtained with each serum sample will fall on a straight line when units of FL inhibited are plotted against log serum concentrations. Determinations can be made by drawing this line for each sample. However, greater accuracy can be gained by considering the results obtained with sera from a group of identically treated animals as a homogeneous population and by calculating the regression line by means of the least squares method (Fisher, 1941). It is then possible to extrapolate and express the results in terms of the mean antifibrinolytic activity per ml. of serum ($[\text{AFL}]_{\text{ml.}}$). For the sake of convenience, results are expressed in the tables in 10^{-3} units. From the standard error of the regression coefficient the standard error of $[\text{AFL}]_{\text{ml.}}$ can readily be calculated. The latter value in turn is the basis for obtaining the standard deviation and the sum of squares of deviations for the number of animals of the group. The significance between mean AFL activities is calculated by means of the *t* test (Fisher and Yates, 1943).

It should be emphasized that the values reported in this paper are valid only under the conditions specified above. AFL activity is influenced by the concentration of FL and, as already mentioned, by the time of contact between enzyme and inhibitor. The kinetics of the FL-AFL reaction are still not clearly understood, and no explanation can be offered at present for the different slopes of the regression lines obtained with different groups of sera. However, this difference gives further support to the view that results observed in the present work are not due to actual changes in the AFL concentration but are due to changes in the ability of AFL to combine with FL.

Blood was collected from guinea-pigs by heart puncture and from rats after decapitation. After clotting, the serum was separated and used either immediately or stored at −25° C. and used within 48 hours.

All surgical removals of glands were performed under ether anesthesia and completeness of the extirpation was checked after death of the animal.[6] The results obtained in animals where removal was incomplete were not included in the calculation of the mean. Adrenelectomized rats were maintained on 1% NaCl in their drinking water.

All injections were made subcutaneously and the dosages expressed per kg. of body weight. When injections were continued for several days, the dose indicated was given daily in a single injection.

RESULTS

A. Guinea-Pigs

1. *ACTH, cortisone, splenin A and splenin B.* In a previous study

[6] Hypophysectomized rats were supplied by Hormone Assay Laboratories, Chicago.

TABLE 1. ACTION OF ACTH, CORTISONE, SPLENIN A AND SPLENIN B ON ANTIFIBRINOLYTIC ACTIVITY OF GUINEA-PIG SERUM

	10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls	65.9	1.2	9	—
ACTH, 50 μg.	79.5	1.8	6	.02–.01
Cortisone acetate, 5 mg.	85.0	1.3	7	.01–.001
Splenin A, 50 μg.	85.7	2.0	8	.01–.001
Splenin B, 1 mg.	30.6	2.0	7	< .001

The dose indicated was given in a single subcutaneous injection.

(Ungar and Damgaard, 1951) it was shown, by means of the viscosimetric method, that sera of guinea-pigs injected with adrenocorticotrophic hormone (ACTH), or with cortisone or with splenin A had an increased AFL activity when compared with the serum of untreated animals. It was also shown that injection of splenin B had an opposite effect on the AFL activity of serum. Table 1 gives the results of experiments in which ACTH,[7] cortisone,[8] splenin A[9] and splenin B[10] were injected in guinea-pigs. The purpose of these experiments was to determine whether the results ob-

TABLE 2. ACTION OF THYROIDECTOMY AND THYROXINE ON ANTIFIBRINOLYTIC ACTIVITY OF GUINEA-PIG SERUM

		10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls		65.9	1.2	9	—
Thyroidectomy	6 days	28.0	1.1	7	< .001
	20 days	40.6	1.8	6	.01–.001
	48 days	54.6	1.9	5	.3–.2
Thyroxine 20 μg.	4 days	56.0	1.8	8	.2–.1
1 mg.	4 days	22.8	1.25	8	< .001

tained by viscosimetry could be duplicated with the spectrophotometric method. As seen in Table 1, the changes in AFL activity were produced in the same direction as observed previously. All of the differences between the mean AFL activity of treated animals was significantly different from that of the controls.

2. *Thyroidectomy and thyroxine.* The AFL activity of serum was tested in seven guinea-pigs at varying intervals after thyroidectomy. Table 2 shows that 6 days after removal of the thyroid gland, the serum AFL was

[7] Lot 79-R kindly supplied by Dr. J. Lesh of Armour Laboratories.

[8] Saline suspension of "Cortone acetate" kindly supplied by Merck Chemical Company.

[9] Dissolved in sesame oil, kindly supplied by Dr. E. Kaiser and Dr. R. T. Rapala of Armour Laboratories.

[10] Crystalline splenin B, prepared by Dr. S. A. Karjala (of this Institute) and dissolved in olive oil.

strikingly reduced and that this reduction became progressively less marked at 20 and 48 days.

It appeared reasonable to assume that treatment of guinea-pigs by thyroxine would produce an effect opposite to the one elicited by thyroidectomy on AFL activity. However, as seen in Table 2, injection of a large dose of thyroxine[11] for 4 days reduced AFL activity even more than did recent thyroidectomy. At a lower dosage, approximating physiological requirements, the reduction of serum AFL was not significant. This paradoxical effect of thyroxine will be discussed below.

3. *Thyroid-stimulating (TSH) and growth hormones (GH) of the pituitary.* It is recognized that thyroidectomy increases the level of circulating TSH either by stimulating its production in the pituitary or through absence of

TABLE 3. ACTION OF THYROID-STIMULATING AND GROWTH HORMONES OF THE PITUITARY ON ANTIFIBRINOLYTIC ACTIVITY OF GUINEA-PIG SERUM

			10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls			65.9	1.2	9	—
TSH	0.5 mg.	3 hours	69.9	1.2	6	—
	1 mg.	3 hours	66.8	1.4	6	—
	5 mg.	1 hour	43.2	1.9	5	.01–.001
		3 hours	36.8	2.6	6	.001
		4 hours	35.1	1.6	5	<.001
		4 days	17.0	1.0	8	<.001
GH	1 mg.	3 hours	43.4	1.1	6	<.001
	5 mg.	3 hours	31.5	1.5	5	<.001
		4 days	30.9	0.9	6	<.001

the normal inactivation of TSH by thyroid tissue (Rawson and Money, 1949). Table 3 shows that injection of 5 mg. of TSH[12] to guinea-pigs reduced the AFL activity of serum one hour after injection, and that the reduction was even more marked after an interval of four hours. When TSH was given daily for five days, serum AFL fell to extremely low levels.

Table 3 shows that the injection of a preparation of growth hormone[13] is also effective in reducing the AFL level. Since neither hormone preparation was pure (the TSH used contained appreciable amounts of GH and vice versa) and since the technique used in the present experiments does not give graded responses, it cannot yet be determined whether the active principle was TSH, GH or a third constituent common to both preparations.

4. *Repeated injections of desoxycorticosterone acetate (DCA).* Selye and Hall (1943) have shown that prolonged administration of DCA to rats

[11] Thyroxine crystals Squibb.

[12] Thyrotropin, Lot 2-D-3, kindly supplied by Dr. I. Bunding of Armour Laboratories.

[13] Lot GH-3 kindly supplied by Dr. I. Bunding of Armour Laboratories.

gives rise to various types of tissue injury; such as, lesions of the heart, arteries, joints, and kidneys. However, it has not been known whether tissue changes induced by DCA were in any way associated with modifications of the AFL activity of serum. Table 4 shows that whereas a single injection of 5 mg. DCA[14] to guinea-pigs failed to produce any change in serum AFL, repeated daily injections caused the AFL activity to fall progressively for about twelve days to a low level, and to increase slowly again even though injections of DCA were continued.

TABLE 4. ACTION OF DESOXYCORTICOSTERONE ACETATE ON ANTIFIBRINOLYTIC ACTIVITY OF GUINEA-PIG SERUM

		10^{-3} $[AFL]_{ml.}$	±S.E.	n	P
Controls		65.9	1.2	9	—
DCA 5 mg.	5 hours	65.6	1.8	6	—
	6 days	50.0	1.3	8	.01
	8 days	37.0	1.1	6	.001
	12 days	33.3	0.9	8	.001
	14 days	38.25	1.2	7	.001
	18 days	47.2	2.1	8	.01
	21 days	50.0	1.9	7	.02
	24 days	51.1	2.3	4	.05

B. Rats

Further study of the mode of action of endocrine factors on the FL-AFL reaction was limited by the difficulty of keeping alive hypophysectomized and adrenalectomized guinea-pigs. Rats were therefore used in the subsequent experiments.

1. *Intact rats.* Table 5 shows that on the whole the action of various hormones on the FL-AFL reaction of the rat is similar to that observed in guinea-pigs. The one exception is the action of thyroxine which increases AFL activity in the rat and decreases it in the guinea-pig. This is probably due to the fact, noted by Selye (1950), that large doses of thyroxine produce stress in rats, thereby stimulating ACTH output of the pituitary. It appears also that cortisone and splenin A increase AFL activity in rats only when given in repeated injections. In contrast, as seen above, guinea-pigs respond to a single injection of these substances.

2. *Removal of glands.* It is seen in Table 6 that hypophysectomy and splenectomy do not affect significantly the serum AFL level in rats. Adrenalectomy and thyroidectomy produce more or less transitory changes. During the first three days after adrenalectomy the AFL level is extremely low, but by the end of one week it is back to normal. After thyroidectomy the same change is observed but a longer interval is required for the return

[14] Desoxycorticosterone acetate U.S.P. kindly supplied by Schering Corporation; injected dissolved in olive oil.

TABLE 5. ACTION OF VARIOUS HORMONES ON ANTIFIBRINOLYTIC ACTIVITY OF RAT SERUM

			10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls			43.85	1.7	8	—
Cortisone	5 mg.	4 hours	41.5	1.9	5	—
	5 mg.	3 days	66.2	1.6	6	.01–.001
DCA	10 mg.	4 hours	44.2	2.1	5	—
		5 days	33.3	1.2	6	.2 –.1
		10 days	34.3	0.7	18	.05–.02
		15 days	31.45	1.4	5	.1 –.05
Thyroxine	0.5 mg.	4 days	68.0	1.2	12	.01–.001
TSH	10 mg.	5 days	29.6	0.9	8	.05–.02
G.H.	5 mg.	5 days	31.7	1.3	6	.1 –.05
Splenin A	0.5 mg.	4 hours	41.3	1.3	6	—
		3 days	66.5	1.5	6	.01–.001
Splenin B	0.5 mg.	3 days	19.3	3.8	6	.01

TABLE 6. ACTION OF REMOVAL OF ENDOCRINE ORGANS ON ANTIFIBRINOLYTIC ACTIVITY OF RAT SERUM

		10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Intact		43.85	1.7	8	—
Hypophysectomy	3 days	46.2	2.1	5	—
	1 week	49.7	1.25	6	—
Thyroidectomy	3 days	17.9	3.2	5	$<.001$
	1 week	20.6	2.1	5	$<.001$
	6 weeks	45.1	3.6	5	—
Adrenalectomy*	3 days	16.4	0.9	5	.01–.001
	1 week	43.5	2.15	5	—
	2 weeks	45.6	1.8	5	—
Splenectomy	3 days	44.6	1.3	6	—
	2 weeks	43.9	1.2	6	—

* Adrenalectomized rats were maintained on 1% NaCl in their drinking water.

TABLE 7. CHANGES OF ANTIFIBRINOLYTIC ACTIVITY IN HYPOPHYSECTOMIZED RATS

			10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls, 1 week hypophysectomy			49.7	1.25	6	—
Thyroidectomy		3 days	46.8	1.8	5	—
Thyroxine	0.5 mg.	4 days	42.4	2.4	4	—
DCA	10 mg.	10 days	45.1	1.3	11	—
TSH	10 mg.	4 days	28.7	1.2	6	$<.001$
GH	5 mg.	4 days	30.85	1.2	7	$<.001$

All animals were hypophysectomized one week before the experiments, except the rats treated with DCA, which were operated 10 days prior to the experiment and DCA treatment started immediately.

to normal levels. A similar behavior was seen in thyroidectomized guinea-pigs.

3. *Hypophysectomized rats.* Table 7 shows that DCA and thyroidectomy do not cause a decrease in AFL activity in the absence of the pituitary. Both pituitary hormones however still elicit the same response as in the intact animal. The action of thyroxine is also inhibited by hypophysectomy, thus confirming the assumption that it was exerted through release of ACTH.

4. *Thyroidectomized rats.* Rats thyroidectomized six weeks prior to the experiment responded in the same manner as the intact animals when submitted to the action of various hormones, with the exception of thyroxine (Table 8). It is probable that in the absence of the thyroid, an overdosage of thyroid hormone did not constitute a severe stress.

TABLE 8. CHANGES OF ANTIFIBRINOLYTIC ACTIVITY IN THYROIDECTOMIZED RATS

	10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls, 6 weeks thyroidectomy	45.1	3.6	5	—
Thyroxine 0.5 mg. 4 days	38.2	1.2	7	.4 –.3
DCA 10 mg. 10 days	21.7	1.1	6	.02–.01
TSH 10 mg. 4 days	31.8	1.0	7	.05–.02
GH 5 mg. 4 days	29.5	1.1	6	.05–.02
Splenin B 0.5 mg. 3 days	26.2	1.9	6	.05–.02

All animals were thyroidectomized six weeks before the experiment.

5. *Adrenalectomized rats.* Table 9 shows that the low AFL level observed three days after adrenalectomy could be restored to normal values and even raised above these by injections of cortisone and splenin A. DCA, however, in spite of its ability to maintain life after adrenalectomy, failed to prevent the fall of AFL.

It is also seen in Table 9 that rats adrenalectomized two weeks prior to the experiments reacted to the injection of various hormones in the same

TABLE 9. CHANGES OF ANTIFIBRINOLYTIC ACTIVITY IN ADRENALECTOMIZED RATS

	10^{-3} [AFL]$_{ml.}$	±S.E.	n	P
Controls, 3 days adrenalectomy	16.4	0.9	5	—
Cortisone 5 mg. 3 days	48.2	1.0	5	<.001
Splenin A 0.5 mg. 3 days	59.0	1.3	5	<.001
DCA 10 mg. 3 days	22.2	1.9	7	.4 –.3
Controls, 2 weeks adrenalectomy	45.6	1.8	5	—
Thyroxine 0.5 mg. 4 days	23.8	2.6	4	.01–.001
DCA 10 mg. 10 days	24.3	2.5	11	<.001
TSH 10 mg. 4 days	33.2	1.9	5	.05–.02
GH 5 mg. 4 days	26.1	1.6	4	.01–.001
Splenin B 0.5 mg. 3 days	31.6	1.2	5	.05–.02

All animals of the first four groups were adrenalectomized 3 days prior to the experiment; those of the last six groups were operated 2 weeks before. All had 1% NaCl in their drinking water.

way as intact animals. It appears therefore that the adrenal cortex is not involved in the cycle of events which causes a fall in the AFL level. The fact that thyroxine decreases AFL after adrenalectomy instead of increasing it is probably due to the elimination of the stress component so that thyroxine acts now in the same way as in guinea-pigs.

TABLE 10. CHANGES OF ANTIFIBRINOLYTIC ACTIVITY IN SPLENECTOMIZED RATS

	10^{-3} $[AFL]_{ml.}$	±S.E.	n	P
Controls, 2 weeks splenectomy	43.9	1.2	6	—
Cortisone 5 mg. 3 days	42.7	1.2	6	—
Splenin A 0.5 mg. 3 days	55.7	2.1	6	.02
Thyroxine 0.5 mg. 4 days	38.0	1.4	6	.3–.2
DCA 10 mg. 10 days	39.7	0.8	5	.3–.2
TSH 10 mg. 4 days	39.8	1.5	6	.3–.2
GH 5 mg. 4 days	42.0	1.4	5	—
Splenin B 0.5 mg. 3 days	23.2	2.0	7	.01–.001

All animals were splenectomized 2 weeks prior to the experiment.

6. *Splenectomized rats.* It was observed previously that cortisone fails to increase AFL in splenectomized guinea-pigs (Ungar and Damgaard, 1951). It is seen in Table 10 that the spleen is necessary also in rats for the action of cortisone on AFL. Splenin A, however, still increases the FL-AFL reaction in the absence of the spleen. Table 10 also shows that splenectomy reduces the response to those stimuli (with the exception of splenin B) which in intact animals cause a fall in AFL level. It therefore seems probable that the spleen is at least partly involved in the mode of action of TSH and GH.

DISCUSSION

According to the results of the experiments just described the endocrine system can act on the FL-AFL reaction in two, opposing ways: ACTH, cortisone and splenin A accelerate the reaction while TSH, GH, thyroxine, DCA and splenin B delay it. The accelerating factors were discussed in a previous paper (Ungar and Damgaard, 1951) and the present experiments confirm the preceding observations in guinea-pigs and extend their validity to the rat.

The endocrine factors involved in the retardation of the FL-AFL reaction pose more complex problems and raise some controversial issues. The original observation of a decrease in AFL caused both by thyroidectomy and thyroxine suggested that the common mechanism might be an increase in the amount of circulating TSH, according to the observations of Rawson and Money (1949). The correctness of this assumption was supported by the fact that thyroidectomy did not cause a decrease in AFL after removal of the pituitary. The action of thyroxine is more complex; the most reasonable interpretation is that in intact rats the stress effect masks a re-

sponse which becomes apparent after adrenalectomy. Hypophysectomy suppresses both the stress effect and the action on TSH or GH. Inhibition of the latter action by thyroidectomy indicates that thyroxine does act on AFL by depressing thyroid function and not by some peripheral mechanism.

The present observations also indicate that DCA decreases the rate of the FL-AFL reaction through the pituitary; the effect could not be observed in hypophysectomized rats. Furthermore, this seems to be a direct action on the pituitary and not through a modification of thyroid function since thyroidectomy did not affect it.

The effect of thyroid function or DCA seems to consist in increasing the available amount of a hormone from the pituitary; this hormone may be either TSH, GH or a third unknown factor present in both hormone preparations used. Because of impurities in these preparations as well as the fact that the test used in the present experiments does not give graded responses, it is not yet possible to identify the active agent.

The results of the present study indicate that the adrenal cortex is bypassed by the chain of events leading to the retardation of the FL-AFL reaction: removal of the adrenals does not alter the response to either DCA or to the pituitary hormones. In contrast, splenectomy appears to inhibit, at least partly, the action of DCA, thyroxine and both TSH and GH preparations. The only substance tested which still decreases AFL after splenectomy is splenin B. This suggests that the tissues producing splenin B, spleen and bone marrow (Ungar, 1947), may constitute the final common pathway for the stimuli which delay the FL-AFL reaction. A possible control of the spleen activity by the pituitary has been reported by several workers who postulated the existence of a "spleen-stimulating" (Perla, 1936) or "lienotropic" (Ruitinga, 1941) factor and a "REStropic" substance, acting on the reticulo-endothelial system (Weitzler-Ligeti and Wiesner, 1938) produced by the pituitary. Whether these factors are in any way related to the action of the pituitary hormones studied in the present work cannot be ascertained from data reported by these investigators.

There appears, however, to be a certain correlation between the present data obtained by using AFL measurements and the results of observations concerning tissue changes induced or facilitated by hormonal treatment. In a series of experiments carried out simultaneously with the present work it was found that the stimuli which increased AFL activity always inhibited inflammation and those stimuli which caused a fall in AFL always accentuated the inflammatory changes (Ungar, Damgaard and Weinstein, 1951).

The relationship between the FL-AFL reaction and the hormonally in-

duced tissue changes described by Selye (1950) is less straightforward. The following findings of Selye are in agreement with the present observations: a) repeated injections of DCA induce certain types of tissue injury in intact rats; b) the same type of lesions can be produced by a lyophilized anterior pituitary extract (LAP) the active principle of which is claimed to be GH (Selye, 1951a); c) DCA fails to produce these lesions in hypophysectomized rats (Selye, 1946) (this claim has not been repeated and has even been contradicted (Selye, 1950)); d) the incidence and severity of certain lesions is increased in thyroidectomized (Selye *et al.*, 1944) or thyroxine-treated (Selye, 1950) rats.

The main difference between Selye's results and the findings reported in this paper pertains to the adrenal cortex. Selye believes that the pituitary (LAP, GH) acts by increasing the production of DCA or similar "mineralocorticoids" in the adrenal cortex and that this may ultimately be responsible for the tissue injury (Selye, 1950, 1951b). The present experiments indicate that DCA acts through the pituitary which releases TSH, GH or an unknown factor and that the effective substance acts independently of the presence or absence of the adrenal cortex.

Some workers believe that DCA induces tissue injury by inhibiting the release of ACTH from the pituitary (Sayers, 1950). This negative action does not explain why hypophysectomy, *per se*, fails to cause tissue damage. It is necessary to assume therefore that DCA, in addition to suppressing ACTH output, causes the pituitary to release some other substance. This would also explain the paradox of hypophysectomized animals being less susceptible to stress than adrenalectomized ones, in spite of the well known fact that the adaptation mechanism does not function in the absence of the pituitary.

The present observations suggest that the "diseases of adaptation" do not consist in an exaggeration of the adaptation syndrome but are due to the intervention of a different set of hormonal mechanisms involving probably a shift in the normal balance of pituitary secretions.

SUMMARY

A new method is described for the estimation of fibrinolysin and antifibrinolysin based on the digestion of fibrinogen by fibrinolysin and the measurement of this action by the ultraviolet absorption of the digestion products. The results of a previous study made with the viscosimetric method were confirmed with the spectrophotometric technique.

Thyroidectomy, large doses of thyroxine and repeated injections of desoxycorticosterone acetate caused a fall in serum antifibrinolysin levels. In hypophysectomized animals, however, none of these stimuli had any appreciable effect. Injections of pituitary thyroid-stimulating and growth

hormone preparations caused a marked decrease in antifibrinolytic activity both in intact and hypophysectomized animals. It is therefore reasonable to assume that the action of the endocrine factors lowering antifibrinolysin levels is mediated by the pituitary.

The presence of the adrenals is not necessary for the action of any of the factors causing a fall in serum antifibrinolysin. There is evidence, however, that the absence of the spleen decreases the reaction. It is possible that all the factors studied act through the release of splenin B, a substance produced by the spleen and bone marrow.

Consideration is given to the bearing of these observations on the interpretation of the endocrine control of inflammation, the mechanism of hormonally induced tissue damage and on the concept of "diseases of adaptation."

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THE NON-CONCOMITANCY OF THE PROGESTATIONAL AND ANTILUTEINIZING ACTIONS OF STEROIDS

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INTRODUCTION

IT IS known that progesterone counteracts luteinization in the guinea-pig (Dempsey *et al.*, 1936; Dempsey 1937), in mice and rats (Selye *et al.*, 1936; Selye 1939). More recently it has been found that intrasplenic ovarian autografts in the castrated guinea-pig offer a very convenient device for the study of the antiluteinizing faculty of progesterone. Hemorrhagic follicles present in these grafts (Ponce de León and Lipschutz, 1944; Woywood and Lipschutz, 1944; Gay, 1944; Lipschutz 1946a; Lipschutz, Ponce de León *et al.*, 1946) were suppressed when oestrogen was administered whereas luteinization was greatly enhanced (Lipschutz, Iglesias, Bruzzone *et al.*, 1948b; Barahone *et al.*, 1950; Bruzzone and Regúlez 1950). However, when simultaneously with oestradiol progesterone was given luteinization also was suppressed (Lipschutz, Iglesias, Bruzzone *et al.*, 1948b; Mardones 1948; Iglesias, Lipschutz and Mardones 1948, 1950). In other words: under the given experimental conditions oestradiol called for luteinization whereas progesterone counteracted it.

The action of oestrogen is not by a direct interference on the ovarian graft (Iglesias, Lipschutz and Rojas 1950) and it is very likely that we are dealing here with various experimental aspects of the control of the hypophyseal gonadotrophic function by steroids.

Two fundamental questions arise here: *first*, has the antiluteinizing action of progesterone any bearing to those autoregulatory events which supposedly underlay the normal sex rhythm in the body of mammals? And *secondly*, is the antiluteinizing action of progesterone concomitant with, or subordinated to the progestational faculty of this steroid? These two questions shall be dealt with in the present paper.

QUANTITATIVE ASPECTS OF THE ANTILUTEINIZING ACTION OF PROGESTERONE

A first step in studying the question whether progesterone may act as an antiluteinizer in the normal body would be establishing what quantities

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of progesterone are still able to counteract luteinization in the intrasplenic graft, and comparing this figure with that of the quantities of progesterone rhythmically produced in the body of the guinea-pig.

In our former experiments quantities of progesterone as large as 270 to 400 μg daily were absorbed from subcutaneously implanted tablets. A very considerable reduction of absorption was obtained by the use of tablets containing but 40% of progesterone mixed with cholesterol (Mardones 1948; Iglesias, Lipschutz and Mardones 1948). Absorption with these tablets was, similarly to what has been seen formerly (Lipschutz, Bruzzone and Fuenzalida 1944; Fuenzalida 1944, 1950), many times smaller than from tablets of pure progesterone (comp. Ia and Ib to ##, Table 1). In work with steroids of slow absorption as ethinyl-testosterone and ethinyl-Δ^5-androstenediol several, up to as much as 20 tablets had to be implanted so as to obtain absorption of sufficiently large quantities (see last column of Table 1). The remaining experimental procedures were in the present experiments the same as described in preceding papers (Lipschutz, Iglesias,

TABLE 1. 132 CASTRATED GUINEA-PIGS WITH INTRASPLENIC OVARIAN AUTOGRAFTS. NECROPSY AT 2 MONTHS AFTER GRAFTING. 41 EXPERIMENTS (x, xx, xxx) FROM FORMER WORK HAVE BEEN INCLUDED FOR COMPARISON

Group	Progestat. action I.U., mg†	M.P.‡	Estradiol per day, μg.	Progestational steroid per day, μg.	Number of animals			Corpora lutea		Number of tablets of progestat. steroid implanted in each animal
					Total (n)	With corp. lut. (n_1)	With large corp. lut.[1]	Average number per animal.[2]	Average of largest diameters[3] mm.	
#		128–129°	26 – 68	0 Progesterone	30	27	22	1.50	1.60	0
##			34 –114	269–400	14	1	0	0.07	0.40	1, pure progest.
Ia[4]	1	128–129°	25 – 52[5]	Progesterone 8– 15	18	9	2	1.40	1.06	½ and 1, 40% progest.
Ib[4]			31 – 54	16– 23	10	3	0	0.80	0.91	1, 40% progest.
	10	158.5–161°		Desoxycorticost. acet.						
IIa			22 – 59	8– 43	7	3	1	1.00	1.30	1, 40% DOC
IIb			24[6]– 48	176–237	5	1	0	0.40	0.95	1, pure DOC
###			17 – 72	180–260	10	2	0	0.30	1.00	1, pure DOC
IIc			22 – 54	283–439	3	0	0	0	0	2, pure DOC
	10	264–266°		Ethinyl-testost.						
IIIa			34 – 64	150–208	10	10	4	1.70	1.21	8 and 12
IIIb			21 – 37	458–967	4	3	3	0.75	1.69	20
	<20	142°		Vinyl-testost.						
IVa			40 – 71[7]	75–244	11	8	5	1.27	1.54	1
IVb			22 – 56	352–659	6	3	1	1.17	1.07	4 and 8
	>10	243°		Ethinyl-androstenediol						
V			25 – 73	82–286	7	7	4	1.43	1.40	4

[1] Diameter of about 1.4 mm.
[2] Sum of C.l. of the group divided by n.
[3] Sum of largest diameters of the group, divided by n_1.
[4] Some animals necropsied at 82 days.
[5] 1 animal with 157 μg.
[6] 1 animal with 9 μg.
[7] 1 animal with 107 μg.
#, ##, ###, taken for comparison from Lipschutz, Iglesias, Bruzzone *et al.*, 1948b, and from Rojas 1949; including also 13 new observations.
† According to Selye and Masson 1943; Massón and Selye 1945; see also Klein and Parkes 1937.
‡ Determination of Dr. F. Fuenzalida, Biochemist of the Department.

Bruzzone *et al.*, 1948b; Barahona *et al.*, 1950). Results are given in Table 1.

As seen from Table 1, half of the animals of group Ia which received 8 to 15 μg. of progesterone per day, had corpora lutea (fig. 1). The average number of corpora lutea per animal was still considerable and only somewhat less than in animals to which estradiol only was administered (see #). However, the diameter of the corpora lutea in general did not reach 1.4 mm. as normal corpora lutea do and as is also the case with intrasplenic grafts when oestrogen alone is administered. Thus it would seem that the quantities of progesterone administered though still unable to prevent production of corpora lutea, counteracted to a certain degree their further growth. In several cases the cells of the corpora lutea were small; however, in no case was the condition of degeneration attained as described in our work with the prolonged action of large quantities of progesterone (Iglesias, Lipschutz and Mardones 1950). In two animals which received as much as 14 to 15 μg. of progesterone per day the corpora lutea attained a diameter as large as 1.6 mm. and were in what seemed to be a perfect condition. On the contrary, in group Ib (Fig. 2), in which 16 to 23 μg. of progesterone were absorbed, only 3 out of 10 animals had corpora lutea; they were small and partly in degeneration.

Comparing the results with 28 animals of group Ia and Ib to those with 14 animals of group ## the conclusion can be drawn that the quantities of progesterone absorbed from a subcutaneously implanted tablet still able to counteract oestrogen-induced luteinization in the intrasplenic ovarian graft are much smaller than one might have thought at the beginning. The antiluteinizing threshold quantity of progesterone was, under the given experimental conditions, probably somewhat more than 20 μg. per day (Ib; Fig. 1 and 2). Indeed, the objection must be made that this figure is based on the assumption that with tablets containing but 40% of progesterone only 40% of the loss of weight is due to absorption of this steroid (Lipschutz, Bruzzone and Fuenzalida 1944; Fuenzalida 1944, 1950). Though this assumption is warranted by experimental findings it might be wise to start, in the following comparative discussion, not from the figures as given for group Ib in Table 1 but from figures representing the whole loss of weight in these experiments, i.e., about 50 μg. per day, instead of 20.

As far as we know no data are available on the quantities of progesterone which are produced in the body of the guinea-pig. According to Hooker and Forbes (1947, 1949a) the blood of the rabbit and mouse contains during the luteal phase between 4 and 8 μg. of progesterone per ml.; similar figures were obtained by these authorities in a monkey and in a woman pregnant since 8 weeks. One may roughly calculate from these figures the total quantity of progesterone present in the luteal phase in the blood of a guinea-pig of say 500 g. Its blood volume is of about 20 ml. (see Dreyer

and Ray 1910, p. 157) which should contain about 80 to 160 μg. of progesterone. Since these figures are based on data obtained with blood of other species than the guinea-pig they are possibly very far from the reality, and as to this our knowledge is much less secure than in the case of oestrogen where the findings of Furlong *et al.* (1949) in the guinea pig could be made use of (Barahona *et al.* 1950). Neither do we know how much progesterone must be daily *produced* in the ovary of the guinea-pig to make the above quantity of 80 to 160 μg. available in the blood; however, it is very likely that a multiple of this quantity has to be released daily by the ovary in the luteal phase. Our results show that a quantity much smaller than 80 to 160 μg. per day is sufficient to counteract or in any case to interfere in luteinization: with about 50 μg. per day, and probably with less than that, luteinization was considerably interfered with (see Table 1, group Ib, and the quantitative objection as discussed in the preceding paragraph). Thus our quantitative results are *not contrary* to the concept that progesterone may partake in an hormonal autoregulatory device through its antiluteinizing action. Indeed, our results do not *prove* it since one may argue that progesterone absorbed continuously during several weeks from a tablet might act differently than progesterone released *rhythmically* for periods of several days only. However, the following comparative statement may be mentioned here. In the guinea-pig about 20 μg. of progesterone per day absorbed continuously from a subcutaneously implanted tablet were sufficient to prevent oestrogen-induced abdominal fibroids (Lipschutz, Bruzzone and Fuenzalida 1944; Bruzzone 1949); quantities not far from that were still antifibromatogenic even when allowed to be absorbed rhythmically only for periods of ten days each (Iglesias, Lipschutz and Nieto 1944).

Whereas these quantitative findings do not allow a definite conclusion as to the applicability of our findings with the antiluteinizing action of small quantities of progesterone to the normal body, they are much in favor of such an applicability. Indeed, for settling the question of the physiological bearing of the antiluteinizing faculty of progesterone it will be necessary to work with a short rated rhythmic action of progesterone.

COMPARATIVE ANTILUTEINIZING ACTION OF DIFFERENT PROGESTATIONAL STEROIDS

Desoxycorticosterone (DOC) shares with progesterone its antiluteinizing faculty (Peñaranda 1947; Lipschutz, Iglesias, Bruzzone *et al.*, 1948b). As in our former work (see ### in Table 1) an almost complete inhibition of luteinization was again obtained with 176 to 283 μg. of DOC acetate per day (groups IIb, IIc). Results in group IIb and ### give full evidence that an antiluteinizing action can be produced with DOC when quantities are administered which are 4 to 10 times greater than the antiluteinizing

quantities of progesterone (Fig. 3 and 4). With smaller quantities of DOC as in group IIa there were 4 out of 7 animals without corpora lutea. It is generally assumed that DOC is only one tenth as progestational as progesterone (Selye and Masson 1943). Our results with the antiluteinizing action of DOC would thus not be contrary to the concept of concomitancy of progestational and antiluteinizing faculties. But such a conclusion is not warranted by our findings with other progestational steroids we have examined.

With quantities of *ethinyltestosterone* (ET) 3 to 10 times greater than those of progesterone (IIIa) all animals had corpora lutea; the number of animals with corpora lutea (n_1) was similar to that in the group with oestrogen alone (see #). Even with quantities of ET as large as 20 times those of progesterone, inhibition of corpora lutea did not take place (IIIb, Fig. 5). Corpora lutea remained in general in a perfect condition (Fig. 6), with the exception of but 1 animal of group IIIa receiving 160 μg. of ET per day. In 1 animal of group IIIb, with 524 μg. per day, corpora lutea were absent. The difference between the groups II and III, that is between the results with DOC and ET, is very striking. And yet ET is as progestational as is DOC (Selye and Masson 1943)!

With quantities of *vinyl-testosterone* (VT) up to 10 times greater than those of progesterone (IVb) an incomplete antiluteinizing action was seemingly obtained: corpora lutea failed to appear in 3 out of 6 cases with 510 to 659 μg. per day. The progestational activity of VT is about 20 times less than that of progesterone.

The difference between *ethinyl-Δ^5-androstenediol* (EA; group V) and DOC is clear cut. There were corpora lutea with as much as 286 μg. per day (Fig. 7). Progestational activity of EA is only slightly smaller than that of ET (Masson and Selye 1945), and nevertheless both these steroids behave so differently from DOC as to antiluteinizing action.

Whereas as already mentioned above the comparative results with DOC and possibly also with VT would not be contrary to the concept of concomitancy of progestational and antiluteinizing actions, results with ET and EA show that the antiluteinizing action is not concomitant with, or subordianted to, the progestational one. ET and EA had no antiluteinizing activity or the latter was smaller than had to be expected according to their known progestational potency. The difference between DOC on one hand, ET and EA on the other is remarkable from various points of view. It has been found that DOC does not act on the uterus of mice when directly applied to the endometrium (Hooker and Forbes 1947, 1949b); its systemic activity is probably due to previous transformation into progesterone (Zarrow *et al.*, 1950). One might then venture that the antiluteinizing action of DOC is due to its special metabolic fate. Nothing is known as

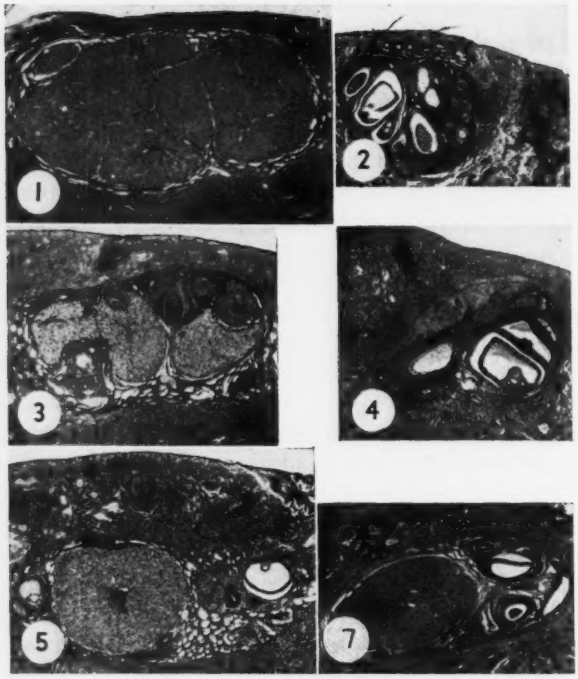

FIG. 1. Intrasplenic ovarian autograft in castrated female guinea pig, 72 days after grafting. The last 52 days 31 μg. of oestradiol and 12 μg. of progesterone per day absorbed from subcutaneously implanted tablets. No hemorrhagic follicles; many corpora lutea. ×20. (CXXVI. 88).

FIG. 2. Intrasplenic graft as in Fig. 1.; 63 days after grafting. The last 47 days 38 μg. of estradiol and 20 μg. of progesterone per day. No hemorrhagic follicles; no corpora lutea. ×20 (CXXVI. 82).

FIG. 3. Intrasplenic graft as in Fig. 1.; 63 days after grafting. The last 46 days 28 μg. of oestradiol and 43 μg. of desoxycorticosterone acetate per day. No hemorrhagic follicles; 3 corpora lutea. ×20 (CXXVII. 172).

FIG. 4. Intrasplenic graft as in Fig. 1; 62 days after grafting. The last 44 days 25 μg. of oestradiol and 201 μg. of desoxycorticosterone acetate per day. No hemorrhagic follicles; no corpora lutea. ×20 (CX. 25).

FIG. 5. Intrasplenic graft as in Fig. 1.; 61 days after grafting. The last 46 days 37 μg. of oestradiol and 967 μg. of ethinyltestosterone per day. No hemorrhagic follicles; large corpus luteum. ×20 (CXXVI. 253).

FIG. 7. Intrasplenic graft as in Fig. 1, 63 days after grafting. The last 51 days 55 μg. of oestradiol and 286 μg. of ethinylandrostenediol per day. No hemorrhagic follicles; large corpus luteum. ×20 (CXXVI. 287).

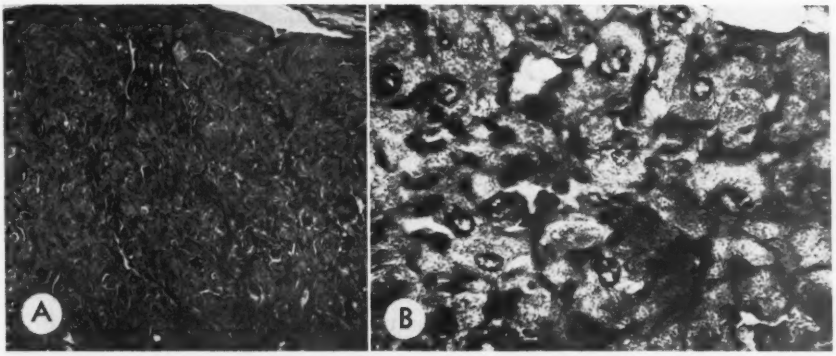

FIG. 6A. Corpora lutea in intrasplenic graft of animals receiving oestradiol and large quantities of ethinyltestosterone. Note perfect condition of corpora lutea.—A. ×90; 961 μg. of ethinyltestosterone per day. (CXXVI. 252)—B. ×400: 967 μg. of ethinyltestosterone (CXXVI. 253).

to the metabolic fate of the other steroids we have examined. ET when directly applied to the endometrium of the rabbit has been found by Höhn and Robson (1950) to be as progestational as progesterone; since it is less effective when entering into the general circulation the authorities assume that it loses part of its progestational activity on account of metabolic changes. It would seem idle to make any tentative suggestion as to the implication metabolic transformations of these and other steroids may have for the fact that the antiluteinizing action of the latter is not in accord with their progestational faculty.

The conclusion referring to non-concomitancy of the antiluteinizing and progestational actions of steroids is coincident with that which has been reached in comparative work on the progestational and antifibromatogenic actions of progesterone and other 3-keto-steroids. The concept of concomitancy which was held originally had to be discarded when new experimental evidence became available (Lipschutz 1946b; Iglesias and Lipschutz 1946; Lipschutz, Iglesias, Bruzzone, *et al.*, 1948a; Lipschutz 1950; Bruzzone *et al.*, 1951). The antiluteinizing action of progesterone is, like its antifibromatogenic action, not subordinated to its progestational action; it is an "independent" action, to apply here again Selye's terminology (see Iglesias, Mardones and Lipschutz 1951; Lipschutz, Iglesias and Mardones 1951).

SUMMARY

The quantities of progesterone necessary for complete prevention of oestrogen-induced corpora lutea in the intrasplenic ovarian autograft of the castrated guinea pig are probably smaller than those which are produced by the ovary in the luteal phase.

The antiluteinizing action of progesterone becomes evident already with quantities smaller than those by which luteinization is completely prevented.

Since these antiluteinizing actions have been obtained with the *continuous* administration of progesterone absorbed from subcutaneously implanted tablets the above statements do not definitely prove an implication of *rhythmically* porduced progesterone in an autoregulatory mechanism by which the sequence of the follicular phases is supposedly determined. But our quantitative findings would not be contrary to such a concept.

Antiluteinizing action is not concomitant with, or subordinated to the progestational one. This conclusion is based on the comparative findings with different steroids. Whereas the antiluteinizing faculty of desoxycorticosterone acetate coincides more or less with what should be expected according to its progestational action in the rabbit test, no antiluteinizing action, or only a slight one, was obtained with large quantities of ethinyltestosterone and ethinyl-Δ^5-androstenediol, though the first one is known to be no less progestational than is desoxycorticosterone acetate.

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NOTES AND COMMENTS

ADRENOCORTICOTROPIN CONTENT OF THE IMMATURE RAT PITUITARY GLAND[1]

The newborn rat differs from the adult in that the pituitary-adrenal system does not respond to a single stress until the animal has reached at least the eighth day of life (Jailer, 1949, 1950). This has been confirmed by Irwin, Buchanan, Longwell, Holtkamp and Hill (1950). It has been postulated that the point of non-reactivity resides in the sphere of the pituitary since the adrenals are capable of responding to exogenous adrenocorticotropic hormone (ACTH) as early as the first day of life. Several possible mechanisms have been formulated to explain this phenomenon (Jailer, 1950). The present report deals with the results of assaying pituitary glands of rats of various ages in an attempt to elucidate the mechanism of non-responsitivity of the hypophysis of the infant rat to stress.

METHODS AND MATERIALS

Rats of the Sherman strain, 4 to 23 days of age, were injected intraperitoneally with sufficient pentobarbital to induce deep anesthesia. The pituitary glands were promptly removed, titurated in physiological saline and the suspension was immediately injected intraperitoneally into 4-day-old recipient rats. Two hours later, the recipient rats were anesthetized, the adrenals removed, carefully dissected and weighed. The assay for ACTH depends upon a fall in adrenal ascorbic acid (Sayers, Sayers and Woodbury, 1948). Adrenal ascorbic acid was determined by the method of Roe and Kuether (1943). In the group of donor rats 4 days old, as many as 4 pituitaries were administered to one recipient. This was done in an attempt to inject equivalent amounts of pituitary tissue. The hypophyses were not weighed because of the fear that due to the consistency of the glands they might become too dessicated. A total of 69 rat pituitaries were assayed in this manner.

RESULTS

The administration of $2\frac{1}{2}$ to 4 four-day-old pituitary glands to one 4-day-old recipient rat did not result in a significant fall in adrenal ascorbic acid. The injection of a suspension of 1 to 2 pituitary glands of 11-day-old rats caused an average fall of but 10 per cent in adrenal ascorbic acid. It was not until the pituitary glands of 18-day-old donor rats were used that there was approximately a 20 per cent decline in the ascorbic acid content of the adrenals of the recipient rats. From previous data, this would indicate the presence of approximately 50 μgm. (equivalent of La-1A) of ACTH. It would appear that there is no detectable ACTH in the pituitary glands of the rats of this strain until they reach approximately 18 days of age. The results are presented in Figure 1.

In order better to understand the relationship between storage and secretion of ACTH, an additional twelve 25-day-old rat pituitary glands were assayed in the following manner: 6 of the animals received 0.1 mg. epinephrine and 6 received 0.1 cc. saline one hour before sacrifice. The pituitaries of the latter group (controls) caused a 40 per cent fall in adrenal ascorbic acid while those of the former (epinephrine injected) but a 17 per cent fall. These results would indicate that ACTH is stored in the gland of these animals and a single stress depletes the hormone content of the gland.

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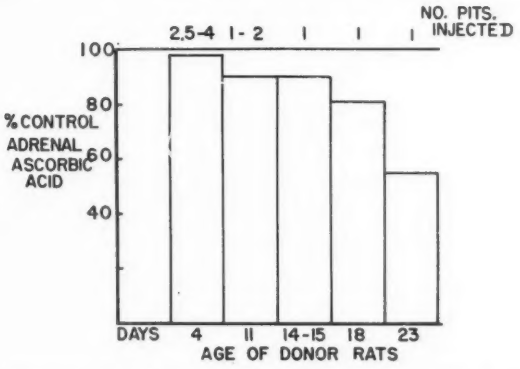

FIG. 1. Percentage fall in adrenal ascorbic acid in recipient newborn rats after the injection of rat pituitary extract.

DISCUSSION

The pituitary-adrenal system of the newborn rat is not activated by a singly applied stress; however, the adrenal gland of such an animal can be stimulated by the administration of ACTH (Jailer, 1950). The above experiments were an attempt to elucidate the reason for the lack of pituitary response to a stress in newborn rats. From the data presented it is possible to speculate that the pituitary glands of these young animals are liberating into the blood stream all the ACTH that the hypophysis is synthesizing and thus there is no intracellular storage. Consequently, when a single stress is applied to rats younger than 18 days, there is little hormone present to be liberated in response to that stress. If a stress is sustained, however, it is entirely possible that synthesis of ACTH is facilitated, and there will then ensue increased liberation of the hormone. Venning (1950) has shown that the premature human will respond to pulmonary atelectasis with an increased excretion of glucocorticoids. This diseased condition is a continuous severe stress as compared to the single stress applied in our studies.

SUMMARY

Pituitary glands of rats 4 to 23 days of age were assayed for ACTH content. No detectable activity was found until the animals had reached 18 days of age. The administration of epinephrine causes a marked fall in the ACTH content of the pituitary gland.

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